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Neoadjuvant Multimodal Therapy in Rectal Cancer

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Fragen ist die Frömmigkeit des Denkens
Martin Heidegger

Contents

Chapter 1

Introduction

1.1 Rectal Cancer: Epidemiological Aspects

Colorectal cancer continues to represent a common malignancy in the developed world. In Flanders, a total of 8,513 cases of invasive colorectal cancer were diagnosed in the period 2000-2001, with 3107 (36.5%) of these situated in the rectum. This incidence is comparable with that in other Western and Northern European countries.[1]
From archival data, it has been shown that actuarial 5 year survival of untreated rectal cancer patients was below 5%.[2] Important advances have been achieved in both preoperative and surgical care, resulting in a postoperative mortality following rectal cancer surgery from more than 50% in the first decades

of the twentieth century to the current mortality rate of less than 2%. Overall survival in colorectal cancer patients is determined by the stage of the disease (Table 1.1). Since most patients present with stage II or III disease, approximately half of all patients will be cured by a combination of surgery and perioperative therapy.

Stage	T	N	M	5YS (%)
0	Tis	N0	M0	
I	T1,T2	N0	M0	73
IIA	T3	N0	M0	59
IIB	T4	N0	M0	
IIIA	T1,T2	N1	M0	44
IIIB	T3,T4	N1	M0	
IIIC	Any T	N2	M0	
IV	Any T	Any N	M1	8

Table 1.1: Overall survival (%) according to stage (TNM 6th edition) of colorectal cancer patients in Flanders in the period 2001-2002. 5YS, 5 year survival.

1.2 Surgery for Rectal Cancer

Unlike the suprapromontorial part of the colon, the rectum is fixed outside the peritoneal lining within the bounderies of the bony pelvis.

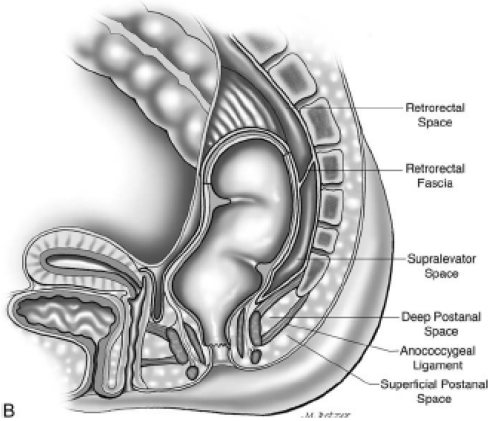

Figure 1.1: Anatomical relationships of the rectum. Adapted from BG Wolff, Ed. The ASCRS Textbook of Colon and Rectal Surgery. Springer, New York, 2007.

The close relationship of the extraperitoneal rectum with nerves, vessels, and other viscera constitutes a challenge for the surgical oncologist, who is expected to acquire a detailed knowledge of the surgical anatomy of the small pelvis aimed not only at complete removal of a rectal tumor but also at the prevention

of a deleterious functional outcome.[3,4]

Historically, rectal cancer surgery has been associated with a prohibitively high local recurrence (LR) rate (up to 40%). The realisation that most of these recurrences were the result of technical error has lead to the concept of 'Total Mesorectal Excision' (TME) as the standard of care in rectal cancer surgery. Although few abbreviations have received as much attention as 'TME' in surgical oncology, the precise meaning of this term is often unknown or misunderstood. The concept of complete removal of the mesentery of the hindgut was given birth by Sir Richard Heald, a surgeon from Basingstoke (United Kingdom). The crux of the TME approach is the observation made together with pathologist Phil Quirke that, in contrast to the tumor in the bowel wall, cancer deposits in the mesorectum can extend distally from the lower border of the rectal cancer.[5-9] Clinical studies have confirmed the need for complete removal of the mesorectum in low lying cancers.[10-12] Another important principle in TME is the so called 'holy plane navigation' with sharp dissection around the tumor and pedicle package (Fig 1.2). Indeed, blunt dissection with 'coning in' of the mesorectum entails the risk of inadvertently entering the mesorectum, an event that may lead to local disease recurrence.

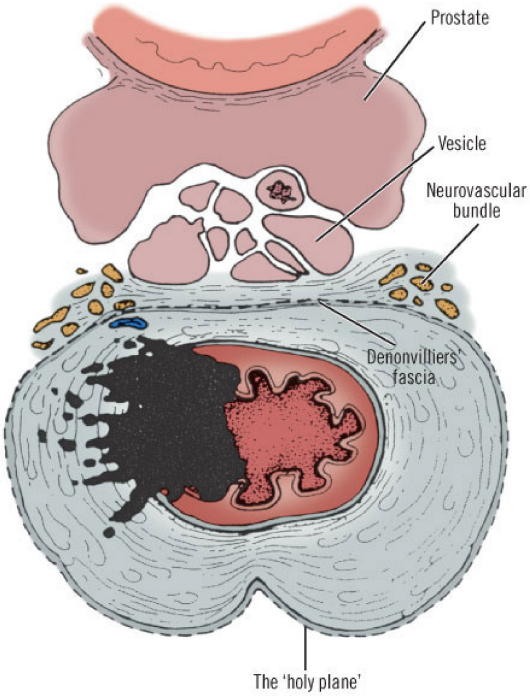

Figure 1.2: Illustration of the resection plane during TME. Adapted from Skandalakis' Surgical Anatomy, McGraw-Hill, Columbus, 2006

There is no doubt that the incidence of locally recurrent rectal cancer has decreased substantially with high quality surgery.[13,14] However, the true incidence is likely underestimated since pa-

tients with metastatic disease often harbour an undetected local recurrence.[15,16] Local recurrence causes substantial suffering for patients, and even with extensive rescue surgery combined with adjuvant therapy the probability of cure is very low.[17-19] Recent national training initiatives in the UK, The Netherlands and Scandinavia have convincingly shown that surgical quality control with implementation of TME improves not only LR rate but also the sphincter preservation rate as well as long term outcome. A similar multidisciplinary project (PROCARE)has recently started in Belgium.

1.3 Perioperative Therapy in Rectal Cancer

In parallel with improvements in surgical technique, adjuvant and neoadjuvant therapy regimens have been developed in order to lower LR rates following surgery. Theoretically, radiotherapy (RT) is aimed at eradication of tumor deposits outside the resection area. An important question is where local recurrences are located in the pelvis. A recent review of clinical and cadaver studies looking at the location of rectal cancer recurrence indicated that the frequency of involvement of subsites in local recurrence patients was as follows: posterior, 49%; lateral, 21%; inferior, 12%, and anterior, 17%.[20] The distance of the tumor from the anal verge is a main determinant of the pattern of lymph node spread.[21] Low lying cancers tend to drain laterally towards internal iliac nodes, and these nodes should

therefore be included in the clinical target volume.[22] The efficacy of RT depends not only on the total dose, but also on the fraction size and treatment duration. To achieve a high probability of eradicating subclinical disease, a biologically equivalent dose (BED)of at least 30 Gray (Gy)is necessary.[23] Since the growth rate of clonogens in rectal cancer is quite short (doubling time 4-5 days), the radiotherapy dose-effect curve shifts to the right when treatment time is prolonged.[24]

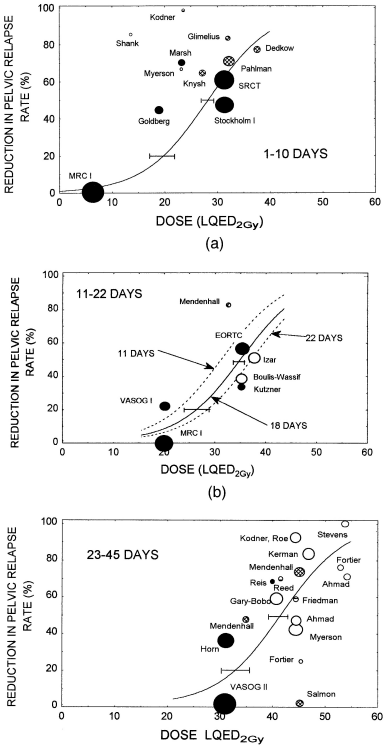

Figure 1.3: Dose response curves obtained from clinical studies using preoperative radiotherapy in rectal cancer. Open circles indicate studies lacking control groups. Circles with a grid indicate studies with historical controls. Black circles indicate randomized trials. The area of the circle is proportional to the number of patients in the combined radiotherapy and surgery groups. Extending the overall treatment time causes a shift of the curve towards higher doses. Adapted from Suwinski R, Taylor JMG, Withers HR. Rapid growth of microscopic rectal cancer as a determinant of response to preoperative radiation therapy. Int J Radiat Oncol Biol Phys 1998;42:943-951.

Preoperative RT has been shown to significantly lower LR rate in seven of the nine randomized trials for which appropriate data have been reported.[25-31] Postoperative RT, on the other hand, resulted in a statistically significant reduction in LR rate in only one of six trials.[32] The only randomized trial comparing preoperative with postoperative RT in rectal cancer showed a lower LR rate with the preoperative approach, even when a higher dose was used postoperatively.[33] Interestingly, the overall survival advantage of preoperative RT is less impressive and smaller than what is obtained with postoperative combined chemotherapy and radiotherapy. A significant overall survival gain was demonstrated only in the Swedish rectal cancer trial[26] and a small Brazilian trial.[34]

Both preclinical and clinical studies have shown that RT does not increase the acute toxicity or anastomotic leak rate provided a careful planning is performed.[35,36] There is, however, a concern regarding the effect of RT on functional outcome. Anorectal function following sphincter preservation is mainly determined by the length of remaining rectum, presence of a reservoir (J-pouch; coloplasty) and preoperative sphincter function. Nevertheless, clinical data suggest that bowel function is significantly worse in patients who underwent RT specifically when large fractions were used.[37,38] Postoperative sexual function has been less well studied, but the available results suggest that RT adversely affects both male and female sexual function following pelvic RT combined with surgery.[39,40] Careful preservation of the hypogastric sympatic branches and lateral parasympatic (erecting) branches during surgery is of

paramount importance in order to preserve sexual and bladder function.[41,42]

The goal of combining RT with chemotherapy is to improve the therapeutic ratio of the combined therapy.[43] This is achieved by spatial cooperation, independence of toxicity, and enhancement of tumor response. The additivity of drug-RT interactions is based on an increased susceptibility of DNA to radiation damage, inhibition of cellular repair mechanisms, cell cycle effects, elimination of hypoxic cells, and prevention of accelerated clonogen proliferation.[44-46] The results from randomized trials performed in the eighties demonstrated that postoperative chemoradiotherapy (CRT) is superior to either postoperative RT alone, chemotherapy (CT) alone or surgery alone.[47,48] As a consequence, this approach was recommended by the National Institutes of Health for all Stage II and III rectal cancers.[49] The experience with preoperative CRT initiated in the setting of unresectable disease, where local control and ultimate R0 resection were noted to be within reach in many patients.[50-52] Extension of this approach to the setting of resectable rectal cancer was therefore a logical step, and multiple phase II trials have demonstrated a promising pathological complete response (pCR)rate with an acceptable acute toxicity following CRT and a 6-8 weeks waiting period.[53-57] Patients with a potentially threatened circumferential resection margin (CRM) or very low lying cancers would theoretically benefit from preoperative CRT. The preoperative approach has a number of advantages over postoperative CRT, including improved radiosensitivity, possibility of downsizing and downstaging, avoidance

of RT toxicity to the small bowel and the anastomosis, and better therapy compliance. These assumptions were confirmed by the German randomized trial comparing preoperative with postoperative CRT using 50.4 Gy with 5-FU.[58] The preoperative approach was associated with a significantly lower LR rate, reduced toxicity, and improved sphincter preservation in a subset of patients deemed to require amputation before enrolment. Overall survival was not significantly different.

Currently, data are available from three randomized trials comparing preoperative RT with CRT in resectable rectal cancer: EORTC 22921, FFCD 92-03, and a Polish trial.[59-65] In the Polish trial, the RT dose and fractionation were different in both study arms: short term RT (5x5 Gy) was compared with with 50.4 Gy + 5-FU. The results of these trials can be summarized as follows: compared to preoperative RT alone, the addition of CT enhances pathological response, lowers local recurrence rate, and may increase sphincter preservation. Acute toxicity is, however, more pronounced while overall survival is not improved by CRT.

1.4 The Prognostic Implications of Tumor Response following Multimodal Therapy

The observation that increasing rates of pCR are obtained with modern CT regimens leads to the interesting concept of local

resection or even organ preservation, analogous to the setting of epithelial cancers.[66] The validity and safety of this approach require critical analysis of two questions: - Does pCR translate into a better outcome? - Can pCR reliably be predicted in patients with a favourable clinical response?

Generally, pathological response following CRT has been shown to be prognostically favourable [67-76] although in one retrospective study pCR following CRT was not related to survival.[77] Some authors have highlighted that even with complete sterilization of the bowel wall, viable tumor might persist in the mesorectum or local lymph nodes.[78-81] The clinical significance of these remaining viable cells is, however, unclear.

1.5 Prediction of Tumor Response to Multimodal Therapy

Accurate prediction of a pCR during or after CRT is therefore of major interest for further therapeutic decisions and prognosis. Neither clinical examination nor routine imaging (CT, MRI, endoscopic ultrasound) are reliable in predicting pCR.[82-86] Similarly, response prediction using a wide array of immunohistochemical markers (p53, p21, p16, Bax, VEGF,thymidylate synthase) has been suggested but as yet the clinical relevance of these markers is uncertain.[87-101] Promising results were recently obtained with gene expression profiling to predict CRT response.[102]

1.6 Response Imaging using FDG-PET

Nuclear medicine imaging using 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) allows non-invasive functional imaging of tumor metabolic activity and is increasingly used to monitor therapy response in several solid cancer types.[103-105] The response to therapy can be quantified by calculating the decrease in standard uptake value (SUV) or by pharmacokinetic modelling using the Patlak model[106]. In rectal cancer, the main interest of FDG-PET concerns the diagnosis of local recurrence.[107] Additionally, several clinical studies have shown that response prediction to neoadjuvant CRT using FDG-PET is superior to EUS in terms of sensitivity and specificity.[108-110] A recent study suggested that FDG-PET is significantly more sensitive (100%) than CT scan (54%) and MRI (71%) in identifying therapy responders.[111] Disadvantages of FDG-PET and combined PET-CT include the health risks and costs of radiation exposure and isotope handling and manufacturing, limited spatial resolution (typically limited to 5-10 mm), limited temporal resolution with inherent motion artefacts, dependence on blood glucose level, limited soft tissue resolution associated with PET-CT, false positive results with inflammatory processes, and dependence of the technique's sensitivity on timing of the scan following completion of CRT (metabolic flare phenomenon).[112]

1.7 Response Imaging using Dynamic Contrast Enhanced Magnetic Resonance

1.7.1 Physiology of Contrast Enhancement in Malignant Tumors

When the size of a growing tumor exceeds 150-200 micrometer, delivery of oxygen and nutients requires a process of development of new vessels termed angiogenesis.[113,114] The tumor vasculature is highly irregular and tortuous and characterized by a number of morphological and functional abnormalities such as deficient pericyte coverage, absence of a basement membrane, deficient intercellular junctions, and presence of cellular lacunae.[115-117] Moreover, tubular structures consisting of both tumor cells and endothelial cells have been noted to exist in tumor tissue. As a result, increased vessel wall permeability resulting from a defective barrier function is one of the best documented properties of the neoplastic vascular bed.[118] Leakiness of the vessel wall is important because it alters the tumor's interstitial tissue pressure and allows macromolecules to enter the interstitial space.

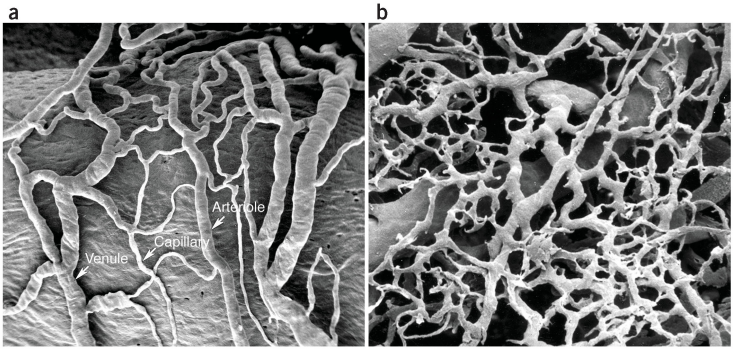

Figure 1.4: Scanning electron microscopic (SEM) imaging of polymer cast of normal microvasculature (a) and tumor microvasculature (b) showing disorganization and lack of conventional hierarchy of blood vessels. Arterioles, capillaries, and venules are not identifiable as such. Reproduced with permission from McDonald DM and Choyke PL. Imaging of angiogenesis: from microscope to clinic. Nat Med 2003; 9: 713-725.

Unlike other diagnostic and imaging modalities, magnetic resonance imaging (MRI) allows to study several aspects of tumor vascular morphology and physiology (blood flow, oxygenation, metabolism, pH) by virtue its superior spatio-temporal resolution and the possibility to employ an array of contrast mechanisms. MRI contrast mechanisms can be broadly classified as intrinsic (endogenous) and extrinsic (Fig 1.5). Endogenous contrast mechanisms such as blood oxygen level dependent (BOLD) contrast and arterial spin labeling rely on endogenous tissue magnetic properties including susceptibility and magnetization transfer. Extrinsic contrast arises from changes in water proton

relaxation behaviour induced by an exogenously administered paramagnetic or ferromagnetic contrast agent (CA). Additionally, MR molecular imaging using targeted CA holds considerable promise.

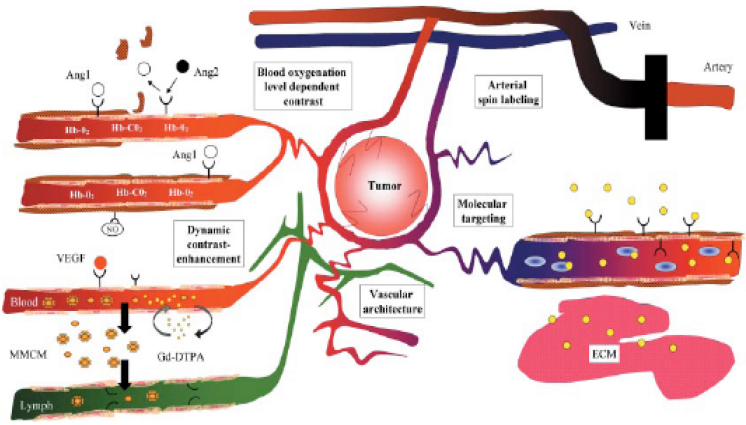

Figure 1.5: Applications of MRI in the study of tumor microvascular physiology. Vascular reactivity can be mapped from changes in blood oxygenation level dependent (BOLD) contrast MRI in response to vasomodulators. Magnetization transfer or arterial spin labeling provides intrinsic perfusion contrast, in which water in a feeding artery is magnetically tagged. Exogenous contrast materials are used in dynamic contrast-enhanced MRI to quantify blood volume, extraction fraction, and vascular permeability. Molecular targeting tags specific moieties on blood components, endothelial cells and the extracellular matrix (ECM). Reproduced with permission from Neeman N and Dafni H. Structural, Functional, and molecular MRI imaging of the Microvasculature. Annu Rev Biomed Eng 2003; 5: 29-56.

Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI, also termed bolus tracking MRI) adds the dimension of time to the anatomical detail of MRI and encompasses rapid

serial image acquisition following intravenous injection of a CA. Observation of the resulting dynamic changes in signal intensity in the tissue(s) under study allows to characterize tissue vascularity, capillary permeability, and volume of the extracellular space.
From a pharmacokinetic point of view, DCE-MRI resembles a dynamic tracer study of a multicompartment physiological system. A 'compartment' here denotes a contained homogeneous physiological entity rather than an anatomical structure. During the DCE-MRI experiment, water soluble CA is injected into the vascular compartment. During the passage of CA through the capillary networks, bidirectional diffusional exchange will occur to and from the interstitial tissue compartment. Since gadolinium based CA does not generally enter the cell, the interstitial compartment is often termed the extravascular extracellular space (EES). The observed intensity and speed of tissue enhancement depends on the vascular supply ('input') and on the extravasation or extraction of CA as it crosses the capillary network. The extraction rate in turn depends on properties of the capillary lining (charge, pore diameter, thickness) and on physicochemical properties of the CA (water solubility, molecular weight, hydrodynamic volume, charge). Leakage of CA can be mathematically described by the Renkin and Crone model of capillary permeability:

$$E = F(1 - e^{\frac{-PS}{F}})$$

Where E represents the extraction rate of CA, F the plasma

flow, and PS the permeability surface product, i.e. the product of capillary permeability and total exchange surface. The importance of this formula lies in the fact that it illustrates the dependence of CA extraction (and thus the observed MRI signal enhancement) on capillary permeability and flow. Two limiting situations can be described:
- high permeability (or small molecule CA): PS>>F; the term

$$1 - e^{\frac{-PS}{F}}$$

approaches to 1 and therefore E $\cong$ F; in this situation extraction is flow limited.
- low permeability (or large molecular weight CA): PS<<F; extraction is permeability limited.
In conclusion: in situations with high capillary permeability (or small CA), the observed tissue enhancement will mainly result from microvascular flow (perfusion) whereas in situations with low permeability (or large CA) enhancement will mainly reflect the capillary permeability. The physiological parameter under study will therefore depend on the choice of CA (small versus large molecular weight agent).

1.7.2 Contrast Agents used in DCE-MRI

Most MRI contrast agents approved for clinical use have a low molecular weight (<500 Da) and, with the exception of the blood brain barrier, diffuse readily across any normal endothelium resulting in a high extraction (50%) during first pass. DCE-

MRI with small molecular weight agents can differentiate tissues (benign versus malignant) based on flow and perfusion characteristics. They are, however, less well suited to study and monitor microvascular permeability.
The clinical importance of neovascular permeability lies in the observations that 1. changes in permeability are a well demonstrated early surrogate marker of angiogenesis [125] and 2. most antitumor therapies induce a 'normalization' of tumor vessels, i.e. the morphology of tumor microvessels changes into a more benign phenotype (less tortuosity, decreased diameter, decreased permeability).[126,127] The prototypic small molecular CA is Gd gadopentetate (Gd-DTPA, Magnevist), a molecule (MW: 547 Da) in which the toxic free Gd(III) is chelated with diethylenetriamine pentaacetic acid.
Several classes of blood pool contrast agents (BPCA) have been developed for MR angiography and functional microvascular tumor imaging, both of which require agents to remain in the normal vascular compartment but to diffuse across hyperpermeable neoplastic endothelium. Although this group of CA is not strictly defined, most authors include CA with a MW range of 5000-50.000 Da.
A first group with intermediate MW (1-60 kDa) is eliminated by glomerular filtration and therefore denoted as 'rapid clearance' BPCA. Examples include Gd-cascade-polymer (Gadomer-17, Schering) and P792 (Guerbet), a macrocyclic compound consisting of a four armed tetracarboxylic chelate. A second class consists of large MW agents composed of Gd ions covalently linked to albumin. The prototypic compound is Albumin-(Gd-DTPA)35, with a MW of 92 kDa. Incomplete elimination

and potential toxicity precludes the clinical use of this agent. Another approach has been the synthesis of agents that reversibly bind to plasma proteins. An example of this category is MS-325 (Schering), a low MW Gd chelate that is 95% bound to serum albumin at equilibrium. A fourth group of BPCA are compounds based on superparamagnetic iron oxide particles (SPIO) usually coated with dextrans. These agents have a MW $>$ 100 kDa, but are rapidly cleared from the blood. A subset of ultra small superparamagnetic iron oxide particles (USPIO) has a significantly longer half-life and holds considerable promise both in lymphatic imaging (by uptake of the compound in lymphatic tissue) and in tumor vascular imaging (by the compound's T1 shortening effect). Even larger BPCA such as liposomal structures or nanoparticles are under active development.

1.7.3 Interpretation and Analysis of DCE-MRI Signal Intensity over Time

Several distinct phases over time can be observed during the DCE-MRI examination (Fig 1.6). When a bolus of paramagnetic, low molecular weight CA passes through a capillary bed, it is transiently confined within the vascular space. The 'first pass' phase includes the arrival of contrast and lasts for a few cardiac cycles (several seconds). Within the vascular space and in the immediate vicinity, paramagnetic contrast media produce magnetic field inhomogeneities that result in a decrease in the signal intensity of surrounding tissues. In most tissues, except the brain, testes, and retina, the contrast agent rapidly diffuzes into the EES. The rate of CA extraction depends on several

Contrast Agent	MW (kDa)	Application
Macromolecular Agents		
Albumin-$(Gd\text{-}DTPA)_{35}$	92	Experimental
Gd-DTPA-Polylysine	36-480	Experimental
Dextrans	52	Experimental
ZK-159560	25.9	Experimental
Rapid Clearance Agents		
Gadomer-17	17	Phase II
P792	6.47*	Phase II
P760	5.29**	Experimental
Serum Albumin Binding Molecules		
Gd-BOPTA	1.058	Clinical use
MS-325	0.96	phase III
B-22956	1.06	Pase II
MP-2269	na	Experimental
Iron Oxide Particles		
Ferumoxtran	100	Clinical
Feruglose	100	Phase II
Ferumoxtran-10	100	Phase II
P-7228	100	Experimental

Table 1.2: Macromolecular contrast agents in preclinical and clinical use. kDa, kilodaltons; na, not available; *molecular diameter 5.05 nm; **molecular diameter 2.8 nm.

parameters including the CA size, capillary density and permeability, blood flow, and size of the extracellular space. After a short time period, diffusion of CA from the EES into the vascular compartment will occur eventually resulting in a kinetic equilibrium. This is followed by a washout phase, during which renal elimination of the CA from the blood results in net flux of CA from the EES into the vascular compartment. Important tumor physiology information can be obtained by selecting a region of interest (ROI) around a clinically relevant tumor zone and by generating a time intensity curve of contrast enhancement in the voxels contained within the ROI. Visual inspection of the resulting curves can assist in differentiating necrotic from viable tumor, or in the evaluation of chemotherapy effects. The time intensity curves can also be quantified resulting in parameters such as onset time (time from CA injection to the arrival in the tissue), initial and mean gradient (slope), maximum signal intensity, washout gradient, and area under the signal intensity over time curve (AUC). This semi-quantitative analysis of tissue enhancement over time has been successfully used in characterization and therapy monitoring of breast and bone tumors (Fig 1.7).

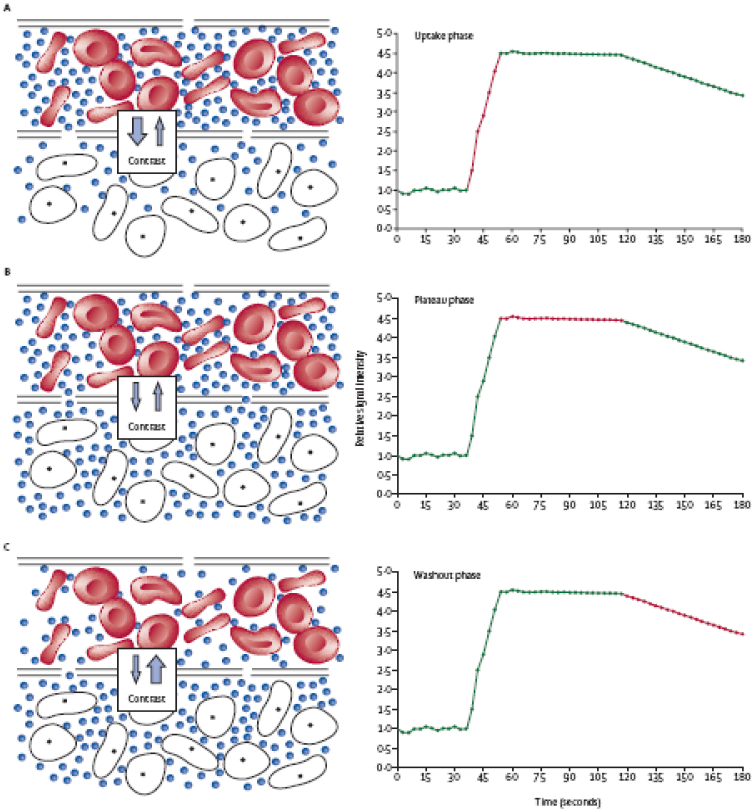

Figure 1.6: Enhancement phases observed during the dynamic MR study. (A) Uptake phase: signal intensity rises above baseline and there is a net leakage of contrast from the blood vessels into the interstitial space. (B) Plateau or equilibrium phase: maximum enhancement with an equilibrium in the movement of contrast between the plasma and extracellular-extravascular space. (C) Washout phase: contrast starts to leave tissue and goes back into blood vessels. Red part of graphs refer to phase in corresponding diagram showing movement of contrast. Reproduced with permission from Zahra MA, Hollingsworth KG, Sala E, Lomas DJ, Tan LT. Dynamic contrast-enhanced MRI as a predictor of tumour response to radiotherapy. Lancet Oncol 2007; 8: 63-74.

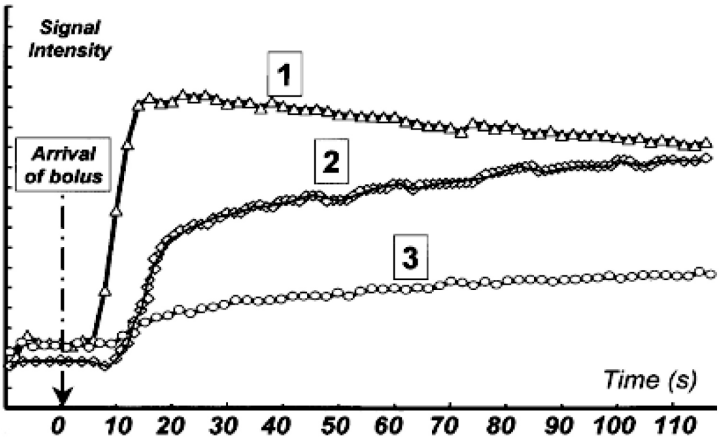

Figure 1.7: Example of DCE-MRI time-intensity curves in the evaluation of bone tumors. The curves display the change in signal intensity over time in three regions of interest. The arrow indicates the time point at which the bolus arrives in the artery. The type 1 curve shows an early and fast enhancement to a maximum signal intensity, followed by early wash-out. This type is seen in arteries, arterio-venous malformation, giant cell tumor, multiple myeloma, and high-grade sarcoma. A type 2 curve shows early and rapidly progressive enhancement, and is found in many benign and malignant musculsokeletal lesions. The type 3 curve shows slow enhancement, indicating low vascularity or slow perfusion, as in myxoma, inactive enchondroma, cavernous hemangioma and muscle, which is often used as reference tissue. Reproduced with permission from Verstraete KL, Lang P. Bone and soft tissue tumors: the role of contrast agents for MR imaging. Eur J Radiol 2000; 34: 229-246.

1.7.4 Pharmacokinetic Modelling of DCE-MRI data

Semiquentitative analysis of the signal intensity over time provides valuable functional information, but does not reflect CA concentration in the tissue of interest and is subject to the variabilities of the MRI machine settings including gain and scaling factors. Pharmacokinetic modelling of DCE-MRI data allows to derive parameters having a physiological meaning such as microvessel permeability, blood volume, and interstitial space volume. By calculating parametric maps based on a pixel by pixel analysis, spatial heterogeneity is greatly reduced compared to ROI analysis techniques. In contrast to semiquantitative techniques, pharmacokinetic modelling is based on CA concentration changes rather than signal intensity changes. The translation of (usually T1 weighted) signal intensity values into CA concentrations is not trivial, and critically depends on the imaging sequence details.

The most commonly used pharmacokinetic models are based on the work of Fick and Kety, who modelled the pulmonary uptake of inert gas as a diffusion process in the context of tracer kinetic hemodynamic measurements.[128,129] This two compartment approach was later applied to DCE-MRI contrast agent kinetics in brain research (blood brain barrier permeability changes in multiple sclerosis).[124,130-138] Several authors have formulated similar two compartment models based on the Kety general model, and recently Tofts et al. proposed a standard nomenclature in order to reconcile the various described methodologies in kinetic modelling of T1 weighted DCE-

MRI(Fig 1.8) .[124]

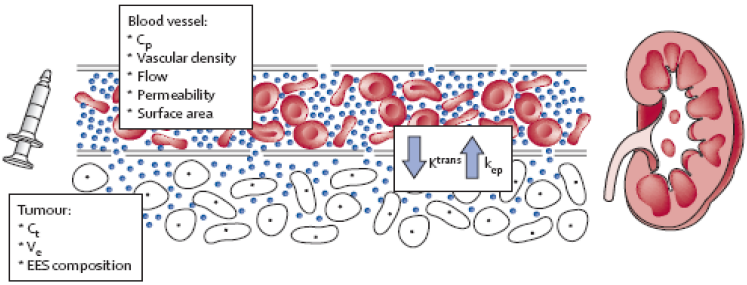

Figure 1.8: Factors that influence the contrast enhancement pattern following bolus injection of CA. Cp=concentration of contrast in plasma; Ct=concentration of contrast in tumour extracellular extravascular space (EES); Ve=fractional volume of EES; Ktrans=volume transfer constant between plasma and EES; kep=rate constant between EES and plasma. Reproduced with permission from Zahra MA, Hollingsworth KG, Sala E, Lomas DJ, Tan LT. Dynamic contrast-enhanced MRI as a predictor of tumour response to radiotherapy. Lancet Oncol 2007; 8: 63-74.

The general two compartment kinetic model describes the change of tissue CA concentration per unit time as

$$v_e \frac{dC_e}{dt} = K^{trans}(C_p(t) - C_e(t))$$

Where Ce denotes the tissue CA concentration, Cp the plasma concentration, Ktrans (dimension min^{-1})the transfer constant, and Ve (dimensionless)the leakage space or EES fractional vol-

ume, i.e. the percentage of the total EES volume available as diffusion space. This formula is derived from the basic concept of mass conservation and states that the tissue concentration is dependent on the concentration difference between the two compartments times a constant (Ktrans). Importantly, this model requires the plasma concentration (vascular input function) to be fitted in the model. Accurate measurement of the arterial input function, perferably in a large vessel feeding the tumor, is not always possible. Some authors have therefore used simulated data or data based on measurements in volunteers as the vascular input function.[137, 138]

This model assumes that intravascular contrast does not contribute significantly to the tissue signal. In tumors, however, blood volume is often high and therefore the measured signal in a tumor voxel will be composed of both extravasated and intravascular contrast. Several authors have attempted to include the effects of a significant vascular component. The solution formulated by Tofts is to add a vascular component: Ct = vpCp + veCe. The final model can then be reformulated as

$$C_t(t) = v_p C_p(t) + K^{trans} \int_0^t C_p(t') e^{\frac{-K^{trans}(t-t')}{v_e}} dt'$$

The Tofts two compartment model assumes bidirectional flux of CA. When only the first pass of the CA bolus through the tissue of interest is analyzed, back diffusion of CA from the EES into the vascular bed can be assumed to be negligible. In this special case, the general model can be simplified to

$$C_t = v_p C_p(t) + K^{trans} \int_0^t C_p(t')dt'$$

This approach is known as the graphical Patlak plot model, which is commonly used in modelling of data obtained with nuclear tracer imaging (PET). When a series of data is plotted with

$$C_t(t)/C_p(t)$$

As the Y axis and

$$\int_0^t C_p(t')dt' \Big/ C_p(t)$$

As the X axis, the slope of the resulting Patlak plot represents Ktrans.[139-141] This model is depending on the assumption that Ktrans is small compared to Ve and does not allow to determine the parameter Ve. It can be attractive in situations where the image acquisition period is limited by technical or patient related circumstances.

In addition to to parameterization of the transfer constant, blood volume and interstitial space, more complex models allow additionally to derive blood flow.[142-144] It should be realized, however, that complex models require high quality data and careful, supervised data fitting in order to avoid spurious results.

1.7.5 Preclinical and Clinical Response Prediction using DCE-MRI

Several preclinical studies have successfully used DCE-MRI to quantify the effects of vascular targeting therapy in tumor models.[145-167] Clinically, DCE-MRI has provided a tool to detect early tumor response to targeted therapy, CT or RT in breast, soft tissue and bone, cervical, and colorectal cancer patients.[168-185] Of note, MMCA are as yet not available for clinical use with the exception of iron particles (SPIO and USPIO). The main clinical interest of these agents is nodal staging using MRI, although in principle they could be used to study tumor blood volume and permeability by measuring T1 effects using DCE-MRI.[186-188]

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Chapter 2

Rationale and Aims

The important issue of locally recurrent disease following rectal cancer resection has been dealt with by improvements in surgical technique (TME) and by the introduction of perioperative therapy regimens consisting of RT either as a single modality or in combination with chemotherapy. The Dutch rectal cancer trial has demonstrated that even with systematic implementation of high quality surgery, many patients will benefit from neoadjuvant RT.[1] The aim of **chapter three** is to provide a systematic literature overview of the published experience with perioperative therapy in resectable rectal cancer. Specific issues addressed in this chapter include RT alone versus CRT, adjuvant versus neoadjuvant regimens, and the importance of RT dose and fractionation. The Scandinavian trials using 5x5 Gy of preoperative RT alone followed by immediate surgery have demonstrated a significant reduction in local recurrence rate.[2]

Since no waiting period after completion of RT is included, tumor shrinkage or downstaging will not occur at a significant level. In patients with a potentially compromised circumferential resection margin and/or tumor located near the sphincter apparatus, tumor downstaging is desirable by intensification of the RT regimen.

In **chapter four**, we compared two regimens of intensified preoperative therapy in locally advanced rectal cancer patients. One group of patients received CRT followed by surgery after a 6 week period, while another group was treated with hyperfractionated accelerated RT (HART) immediately followed by surgery. Both groups were compared in terms of local and systemic side effects, recurrence rate, and long term survival.

Assessment of the degree of response to neoadjuvant therapy is important because it influences therapeutic decisions such as the extent of surgery but also has been shown to influence long term outcome.[3,4] Early detection of tumor response allows to timely abandon or upgrade therapy in patients who fail to respond. Current imaging modalities to assess therapy define response in morphological terms, i.e. downsizing or downstaging. Since tumor shrinkage is often a late event and tumor response does not always correlate with changes in size, functional imaging strategies have been put forward to visualize early therapy effects. Metabolic imaging using PET or PET-CT has been shown to allow early prediction of therapy response to preoperative CRT.[5] Along with the metabolic activity of neoplastic cells, the ultrastructure and function of the tumor microvascular bed could allow early detection of therapy effects.

It has indeed been recently demonstrated that angiogenesis tar-

geting therapies induce an early 'normalization' of the tumor vascular bed.[6] The observed changes after therapy include increased pericyte coverage, decreased vessel diameter and density, and decreased permeability. Recent evidence suggests that this 'vessel normalization' is also observed after RT.[7] Functional aspects of the tumor microvascular bed and the changes induced by antitumor therapy can be examined by DCE-MRI using Gd based CA.[8,9] In **chapter five**, we examined early changes in the tumor microvascular bed following 5x5 Gy or RT in a rat colorectal cancer model. Functional imaging was performed with DCE-MRI using P792, a rapid clearance BPCA. The functional imaging data were fitted in a two compartment pharmacokinetic model and the calculated vascular parameters compared with histology.

Response to preoperative RT is variable and largely determined by the tumor's radiosensitivity. One of the best studied physiological parameters having an influence on radiosensitivity is tissue oxygenation. Numerous clinical studies have demonstrated a strong association between tumor hypoxia, radioresistance, and poor therapeutic outcome.[10] Strategies to improve tumor oxygenation during therapy have therefore been actively pursued. Since the blood's oxygen content is mainly determined by the haemoglobin level and many cancer patients are anemic, administration of erythropoietin (EPO) theoretically could result in improved radiosensitivity and hence a better outcome. In clinical trials using EPO, however, the expected improved outcome has not always been observed.[11,12] Moreover, recent preclinical data suggest that EPO itself might influence cancer growth and tumor radiosensitivity, a finding further corrobo-

rated by the demonstration of the EPO receptor on many cancer cell types.[13] Tovari et al. demonstrated that EPO administration in a xenograft model significantly increased the proliferation index of the tumor-associated endothelial cells and the size of CD31-positive intratumoral blood vessels, whereas the pericyte coverage became fragmented.[14] The effect of EPO on the response of tumor microvessels to RT is unknown. In **chapter six**, we aimed to examine the effect of recombinant human EPO on tumor microvascular morphology and function following 5x5 Gy of RT in a rat colorectal cancer model. A similar DCE-MRI imaging method was used together with histology and immunohistochemistry.

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Chapter 3

Preoperative Combined Modality Therapy in the Management of Locally Advanced Rectal Cancer

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3.1 Abstract

Aims: to review the use of preoperative combined modality therapy (CMT, chemotherapy with radiotherapy) in the management of resectable rectal cancer.
Methods: a systematic search was performed on preoperative CMT and rectal cancer. Additional information was retrieved from hand searching the literature and from relevant congress proceedings. We addressed the following issues: phase II studies of preoperative CMT, preoperative radiotherapy (RT) alone versus preoperative CMT, preoperative versus postoperative CMT, functional outcome and pathologic downstaging after CMT, prediction and importance of complete response to CMT.
Results: preoperative CMT results in an average pathological complete response (pCR) rate of 18.5% in phase II studies. Compared with preoperative RT alone, the addition of CT significantly improves tumour response but not overall survival while acute toxicity increases and the effect on sphincter preservation is at present unclear. Preoperative CMT has been proven to be superior to postoperative CMT in a German multicenter randomized trial. The scarce available data suggest that the addition of CT might worsen anorectal function compared to preoperative RT alone. Although a significant pathological response is prognostically favourable, the clinical and imaging tools available at present do not allow to accurately predict pCR in clinical complete responders confirming the indication for surgery in this subgroup.
Conclusions: preoperative CMT enhances tumour response and could therefore have a role in patients with possibly in-

vaded resection margins or low lying cancers, although both acute toxicity and anorectal function are worse compared to RT alone. The final results of ongoing randomized trials will more accurately establish the role of preoperative CMT in resectable rectal cancer patients.

3.2 Introduction

The last decade has seen important changes in the management of resectable rectal cancer. Advances in both surgical technique and adjuvant therapies have markedly reduced the incidence of recurrent disease while offering sphincter preservation to more patients with low lying cancers. Many questions remain, however, and the increasing complexity of rectal cancer therapy justifies a tailored approach guided by a multidisciplinary team. The aim of this overview is to focus on new developments and current controversies surrounding preoperative rectal cancer management. More specifically, the following topics will be addressed: neoadjuvant versus adjuvant therapy; neoadjuvant chemotherapy versus chemoradiation (CRT), clinical significance and prediction of tumour response, and the concept of organ preservation.

3.3 Methods

A systematic search was performed in the English literature. The following electronic databases were searched: Cochrane

Central Register of Controlled Trials; ISI Web of Science (Science Citation Index, Current Contents) from 1975 until september 2005, and Embase.com. The search was performed using both MeSH (Medical Subject Heading) terms and free text terms:
-MeSH: "Rectal Neoplasms"[MeSH] AND "Radiotherapy"[MeSH] AND "Drug Therapy"[MeSH]. -Free text terms: rectal, rectum, cancer, adenocarcinoma, neoplasm, radiotherapy, irradiation, chemotherapy, chemoradiation, radiochemotherapy, combined modality, multimodal. No formal meta-analysis was performed. Additionally, published proceedings of ASTRO (American Society for Therapeutic Radiology and Oncology) and ASCO (American Society of Clinical Oncology) were searched from 2000 until 2005.

3.4 Results

3.4.1 TME: a Redefined Role in the Adjuvant Therapy Era

Due to the specific anatomy and biology of rectal cancer, surgery alone historically has been associated with local recurrence in up to one in four patients. Neoadjuvant RT has been shown to significantly decrease local recurrence rates provided a biologically equivalent dose of at least 30 Gy is administered.[1] Remarkably, in only one of the fourteen randomized trials (the Swedish Rectal Cancer Trial) comparing RT followed by surgery with surgery alone was a significant survival benefit demonstrated.[2]

The pooled isolated local recurrence rate in the surgery alone arm of these randomized trials (which predate modern developments in surgical technique) was 17%.

The development of sharp total mesorectal excision (TME) for low lying cancers was essentially based on the finding that tumour deposits were harboured by the mesorectum distally from the lower edge of the bowel cancer.[3-5] Pioneered by R Heald, the results of TME have been paralleled not only by other single centre experiences but, importantly, by nationwide training programs resulting in a dramatic lowering of local recurrence rates.[6-8] The question therefore arose, whether routine application of optimal surgery would obviate the need for neoadjuvant RT in stage II or III rectal cancer. One important answer to this question came from the Dutch Rectal Cancer Trial (CKVO 95-04), which randomized patients to 5x5 Gy of preoperative RT followed by TME versus TME alone after a nationwide surgical training programme.[9] The local recurrence rate at two years was 2.4% in the RT-plus-surgery group and 8.2% in the surgery only group ($p<0.001$), with no difference in overall survival.

The recently presented 5 year results of the Dutch TME trial show a persistently significant difference in local recurrence rate (5.8% versus 11.3%, $p<0.001$).[10]

Interestingly, subgroup analysis demonstrated that RT was effective in tumours of the middle third (5-10 cm) of the rectum, but not in tumours of the upper (10-15 cm) or lower (0-5 cm) third. This suggests that upper third cancers are adequately treated with surgery alone, while the relative inefficacy of RT in lower third cancers may be explained by a different lymphatic spread (towards lateral lymph nodes outside the mesorectum)

and by a higher incidence of invaded resection margins in low lying cancers.[11,12]

3.4.2 The Rationale for CMT - Preoperative Phase II Studies

For advanced stage and/or low lying cancers, preservation of sphincter function is an important goal even if the quality of life advantages of this approach are still debated.[13] In the randomized trials comparing preoperative RT with surgery alone that have used a biologically equivalent dose of at least 30 Gy, the rate of sphincter preservation did not differ between both groups (Table 1).

Table 1
Sphincter preservation rate and pathology results in randomized trials comparing pre-operative radiotherapy (biologically equivalent dose ≥ 30 Gy) followed by surgery with surgery alone

Ref.	Total dose/DPF (Gy)	Interval (weeks)	%SP		%Stage I or II		*p*
			RT	Control	RT	Control	
72	45/1.8	4	na	na	71	31	0.065
73	34.5/2.3	1.5	14	21	64	66	ns
74	40/2	4	32	36	71	41	0.0002
75	20/5	1	na	na			
76	25/5	1	36	36	58	59	ns
2	25/5	1	44	41	68	59	0.008
9	25/5	1	65	67	59	56	ns

SP, sphincter preservation; DPF, dose per fraction; RT, radiotherapy; na, not available.

In three of these trials a significant preponderance of early stage pathological stages was noted in the RT group. A pCR was, however, rarely achieved. The rationale to combine chemotherapy with RT is firmly grounded in the radiobiological principles of spatial cooperation and enhancement of tumour response.[14]

In rectal cancer, CRT was first applied in the adjuvant (postoperative) setting and evaluated in two randomized trials. The Gastrointestinal Tumor Study Group (GITSG) GI-7175 study randomized 227 patients to four adjuvant treatment arms: (1) no adjuvant therapy, (2) chemotherapy only (fluorouracil with semustine), (3) RT only, and (4) CRT. Overall 5 year survival was 59% in the CRT arm versus 43% in the no adjuvant therapy arm (p=0.01) and 52% in the RT alone arm (p=NS). Interestingly, local recurrence rates did not differ significantly over the 4 treatment arms.[15,16] The North Central Cancer Treatment Group (NCCTG) 794751 trial randomized 204 patients to either adjuvant RT alone or adjuvant CRT. Both local control and overall survival were significantly better with adjuvant CRT.[17] The results of both trials lead the National Institutes of Health (NIH) to recommend postoperative CRT as preferred therapy in stage II and III rectal cancer.[18] Clinical experience with preoperative (neoadjuvant) CRT initiated in the setting of unresectable disease, where it was shown that significant downstaging could be achieved resulting in eventual R0 resection in many patients.[19-21] In the setting of resectable disease, neoadjuvant CRT has been evaluated in a large number of phase II studies using different chemotherapy regimens (Table 2).
The mean pCR rate achieved in these studies was 18.5% (95%CI: 15.6%-21.4%) with a mean sphincter preservation rate of 58.7% (95%CI: 51.7%-65.7%). Treatment related toxicity was usually acceptable (grade 3 toxicity: 2.8%-28%) whereas postoperative morbidity (including anastomotic leaks) was not different from surgery alone series. In one phase II study a prohibitive toxicity and adverse effects on quality of life were related to neoadjuvant

Table 2
Phase II trials (published as full paper) of pre-operative combined modality treatment (CMT) in locally advanced resectable rectal cancer

Ref.	Year	*n*	RT total dose/ DPF (Gy)	Chemotherapy	%SP	%pCR	% LR
77	2005	37	50.4/1.8	5-FU, irinotecan	64	22	7
78	2004	140	45/1.8	5-FU, leucovorin	75	24.3	10
79	2004	30	50.4/1.8	Raltitrexed, oxaliplatin	93	30	8.3
80	2004	54	50.4/1.8	Raltitrexed	83.3	24	3.7
81	2004	94	45/1.8	Uracil/tegafur	47.2	9	5
82	2003	27	45/1.8	5-FU, LFA	33.3	22.2	4
83	2003	40	50/1.8	5-FU, oxaliplatin, LFA	62.5	15	na
84	2003	36	45–54/1.8	5-FU, mitomycin C	52.8	2.8	7.7
85	2003	32	50.4/1.8	5-FU, CPT-11	50	37	na
86	2003	36	50.4/1.8	5-FU, cisplatin	75	19.5	na
		32	50.4/1.8	5-FU, mitomycin C	93.8	21.9	na
87	2003	32	50.4/1.8	Capecitabine, oxaliplatin	37.5	19	na
88	2002	32	45/1.8	5-FU, doxifluridine	81.2	12.5	na
89	2002	45	50.4/1.8	Capecitabine, leucovorin	87	31	na
65	2002	41	50.4/1.8	Uracil/tegafur, leucovorin	63	15	8
90	2002	50	45/1.8	5-FU	na	8	na
45	2002	165	37.8–50.4/1.8	5-FU, mitomycin C, cisplatin	73	10.3	na
91	2001	43	45–50.4/1.8	5-FU	58	na	4.6
		43	45–50.4/1.8	Tegafur	62.8	na	2.3
92	2001	68	45/1.8	5-FU	57	25	3
93	2001	82	50.4/1.8	5-FU	61	16	na
22	2001	42	45/1.8	5-FU, leucovorin	39	16	na
94	2001	141	45–50.4/1.8	5-FU, cisplatin	46.8	24	5
95	2001	37	54/1.8	5-FU	76	14	6
96	2001	72	50.4/1.8	5-FU, leucovorin	68	13	1
97	2000	22	45/1.8	5-FU	47	5.3	na
98	2000	39	45/1.8	5-FU, leucovorin	64	15	5.7
66	2000	66	45/1.8	5-FU, leucovorin	58.3	15.6	3.4
99	2000	45	52.5/1.8	5-FU	79	31	na
100	2000	51	40–53/1.8	5-FU, leucovorin	84.3	15.7	0
101	1999	117	45/2	5-FU	59	27	na
102	1998	88	30–45/1.8-2	5-FU, folinic acid	25	7	12
103	1995	20	45–61/1.8	5-FU, leucovorin	25	35	11
104	1995	77	45/1.8	5-FU	67.5	29	3.9
105	1994	34	37.8/1.8	5-FU, mitomycin C	66.7	15	na
106	1994	31	55.8/1.8	5-FU	68	10	13
107	1993	20	45–50/na	5-FU, leucovorin	20	20	10
108	1993	46	40/2	5-FU, mitomycin C	23.9	4	39.1
109	1988	64	40/1.7–1.8	5-FU, mitomycin C	3.1	12.5	na

RT, radiotherapy; SP, sphincter preservation; pCR, pathological complete response; LR, local recurrence rate; DPF, dose per fraction.

CRT using 5-FU with leucovorin.[22] Overall, the results of these phase II studies suggest a clear rationale for preoperative CRT in resectable rectal cancer with a potentially endangered CRM and/or low lying tumours amenable to downsizing.

3.4.3 Preoperative RT alone versus CRT

In the setting of unresectable disease, 4 small randomized trials comparing preoperative RT versus CRT were performed.[23-26] Taken together, the conflicting results and methodological flaws of these trials do not allow to draw a conclusion regarding the superiority of CRT over RT alone. For resectable disease, the results of both nonrandomized and randomized trials are summarized in Table 3.

Table 3
Studies comparing pre-operative radiotherapy alone with pre-operative chemo-radiotherapy in rectal cancer

Ref.	*n*	RT total dose/DPF (Gy)	Chemotherapy	SP%	pCR%	LR%
Historical comparisons						
110	37	45/1.8[a]	5-FU, cisplatin	35 ($p<0.01$)	5	3 ($p<0.01$)
	36	45/1.8		8	6	33
111	15	40–46/na	5-FU, LV, dUR	26.6 ($p=0.02$)	13.3 ($p=$NS)	na
	27	40–50/na		3.7	0	na
112	207	45/1.8	5-FU	39 ($p<0.01$)[b]	23 ($p<0.01$)	na
	196	45/1.8[c]		13	5	na
Randomized studies						
113	124	34.5/2.3	5-FU	12.9 ($p=$NS)	4.8 ($p=$NS)	na
	121	34.5/2.3		6.6	2.5	na
114	157	50.4/1.8	5-FU, leucovorin	58	16 ($p<0.01$)	na
	155	25/5		61	1	na
115	370	45/1.8	5-FU, folinic acid	52.6	11.7 ($p<0.01$)	na
	363	45/1.8		51.7	3.7	na
116, 117	506	45/1.8	5-FU, leucovorin	55.6 ($p=0.05$)	14 ($p<0.01$)	na
	505	45/1.8		52.4	5.3	na

RT, radiotherapy; SP, sphincter preservation; pCR, pathological complete response; LR, local recurrence; DPF, dose per fraction; dUR, doxifluridine.
[a] Eleven patients additionally treated with intraoperative RT.
[b] In patients with tumours ≤6 cm from the anal verge.
[c] In this group eight patients received CT and 26 patients received 20 Gy in five fractions.

From the EORTC 22921 and the French FFCD 9203 trials, only preliminary data are available. The addition of CT significantly improved tumour response, although overall survival was unaffected and treatment related toxicity worsened. Interestingly, the increase in pathological downstaging was translated into enhanced sphincter preservation in only 1 of the 4 randomized studies. This finding may be explained by the reluctance of surgeons to perform a low anastomosis with tissue that was cancer invaded before initiation of CRT.
The ongoing Trans-Tasman Radiation Oncology Group (TROG) 0104 study will analyze local recurrence rate after short course preoperative RT versus long course preoperative CRT with continuous infusion 5-FU; accrual is expected to be complete around mid 2005. Preliminary toxicity data did not show a difference in adverse events or perioperative mortality rate between the treatment arms.[27]

3.4.4 Preoperative versus Postoperative CMT

The often cited advantages of the preoperative approach include enhanced activity in well oxygenated tissue, better treatment compliance, reduced early and late toxicity, and enhanced sphincter preservation by tumour downstaging and downsizing. Three large randomized trials comparing preoperative versus postoperative CRT were initiated.
Two American trials, Radiation Therapy Oncology Group (RTOG) 94-01 and National Surgical Adjuvant Breast and Bowel Project (NSABP) R-03, were closed prematurely due to insufficient accrual. Partial results from NSABP R-03 suggested that the pre-

operative approach resulted in increased sphincter preservation with marginally higher treatment related toxicity (Table 4).

Table 4
Randomized studies comparing pre-operative with post-operative combined modality treatment in rectal cancer

Ref.		*n*	RT dose/DPF	Chemotherapy	SP%	pCR%	LR%
118	Preop	421	50.4/1.8	5-FU	69	8	6 ($p<0.01$)
	Postop	402	50.4/1.8	5-FU	71	–	13
119, 120	Preop	130	50.4/1.8	5-FU, LV	50[a] ($p=0.01$)	10	na
	Postop	137	50.4/1.8	5-FU, LV	33[a]	–	na

RT, radiotherapy; SP, sphincter preservation; pCR, pathological complete response; LR, local recurrence; LV, leucovorin.
[a] After total inclusion of 116 patients.

The recently published German multicenter trial demonstrated that preoperative CRT was associated with improved compliance, increased local control and reduced toxicity (Table 4). Sphincter preservation rate did not differ between both treatment arms. However, significantly more sphincter saving procedures were performed in a subgroup of patients with low lying cancers judged before randomization by the surgeon to require amputation. Both the NSABP R-03 and German trial failed, however, to show a survival advantage associated with the preoperative approach.

3.4.5 Is There Still a Place for Short Term, High Dose RT?

Although the 5x5 Gy schedule has proven its efficacy in large, conclusive randomized trials, it has been criticized due to alleged toxicity and the unability to achieve tumour downsizing.[28,29] An increase in treatment related morbidity and mortality was

observed in the early Stockholm I and II trials, in which a large volume of the pelvis and abdomen were included in the radiation field.[30] With appropriate small bowel shielding and treatment planning, preoperative 5X5 Gy of RT did not worsen postoperative mortality in the Swedish and Dutch rectal cancer trials. Functional outcome data from the Scandinavian and Dutch trials indicate that, compared to surgery alone, preoperative 5x5 Gy of RT comes at a price.[30-32] On the other hand, data from the Dutch rectal cancer trial suggested that, although 5x5 Gray of RT lead to more sexual dysfunction and slower recovery of bowel function, health related quality of life was not significantly affected by preoperative RT.[33] Moreover, short term RT is convenient for the patient, and does not delay surgery. The 5x5 Gy regimen is aimed at eradicating microscopic disease outside the resection field. An important limitation of the short term regimen immediately followed by surgery is that it is unable to achieve tumour downsizing, a phenomenon observed when a waiting period of several weeks is allowed.[34,35] Furthermore, data from the Dutch rectal cancer trial indicate that preoperative 5x5 Gy of RT cannot compensate for positive circumferential resection margins(CRM).[36] Involvement of the CRM can be accurately predicted by preoperative high resolution thin slice MRI.[37,38]

Taken together, these data suggest that preoperative 5x5 Gy of RT with immediate surgery is a safe and (cost)effective regimen provided adequate RT planning is performed. For patients in whom the CRM is at risk or sphincter preservation is an issue, a longer waiting period and/or a longer RT regimen should be considered. At present, no randomized studies are available

comparing preoperative short term with long term RT alone. The ongoing Stockholm III trial comparing preoperative 5x5 Gy with immediate surgery, 5x5 Gy with delayed surgery, and 25x2 Gy with delayed surgery will provide further important data. Interim results from this important trial including quality of life data will soon be available (Dr B Cedermark, Karolinska Institute, Sweden).

3.4.6 Prediction and Prognostic Importance of Response to CRT

Since preoperative CRT does not improve overall survival compared to preoperative RT alone or postoperative CRT, the prognostic significance of pathological response to CRT is unclear. Several authors have specifically analyzed the relationship between presence and degree of pathological response and long term outcome parameters. The degree of pathological response is usually expressed using a semi-quantitative scoring system such as the tumour regression grade (TRG).[39,40] While some studies demonstrated an improved long term survival in responders, others found long term outcome to be related not to treatment response but to pretreatment clinicopathological variables such as T stage and differentiation.[41-47] Taken together, however, these studies do suggest that a significant response to CRT is a favourable prognostic factor. In order to avoid potential toxicity in non responding patients, pretreatment prediction of response could be an important tool to tailor preoperative therapy. A number of histological and molecular markers such as Bax expression, p53 nuclear staining, and thymidylate

synthase expression have been identified as predictors of pathological response.[48-50] Moreover,the powerful tool of gene expression profiling using microarrays was recently shown to be of value in discriminating responders from non responders.[51] Finally, functional imaging techniques using dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) or nuclear scintigraphy with specific markers have been used to combine morphological data with tumour response prediction.[52,53]

3.4.7 Significance of Complete Clinical Response and the Concept of Organ Preservation

Modern CRT schedules result in a pCR rate exceeding 20%, and it is likely that this percentage will increase in the years to come with the addition of targeted therapy. As a consequence, the concept of organ preservation with nonoperative management in selected patients with a clinical complete response (cCR) has emerged. The provocative study of Habr-Gama et al. compared operative versus nonoperative treatment for stage 0 distal rectal cancer following CRT.[54] In this retrospective comparison of cCR patients who were observed versus clinical partial response patients who underwent surgery and found to have a pCR, long term survival and local recurrence rates were found to be similar. However, several drawbacks concerning nonoperative management in cCR patients should be considered. First, neither clinical examination nor endoscopy or endorectal ultrasound were able to accurately predict a pCR in a number of clinical studies.[55,56]
In a recent study, the negative predictive value of functional nu-

clear imaging with PET in the prediction of pCR was only 43%, i.e. in 19 of 51 patients with a negative PET scan did the specimen contain residual cancer.[57] Second, pathology studies have shown that the mesorectum can harbour residual cancer deposits in patients with a complete sterilization of the bowel wall, indicating a differential response to CRT in these 2 anatomical entities that renders biopsies of the bowel wall insufficient as a basis for therapy decision.[58-60] The risk of nonoperative management was illustrated by the paper of Nakagawa et al., who found locally recurrent disease in 8 out of 10 cCR patients managed expectantly.[61] Nonoperative management therefore is only acceptable in patients refusing or unfit for surgery or in the context of a prospective clinical trial.

3.4.8 Anorectal Function after Preoperative CRT and Sphincter Preserving Surgery

Anorectal function is known to be disturbed by a very low colorectal or coloanal anastomosis, and is further compromised by preoperative RT.[62-64] Little data are available in the literature concerning the additional effects of CRT on anorectal function following sphincter preservation. In a small number of phase II studies, the functional results reported suggest that CRT does not in itself significantly worsen functional outcome.[65,66] A prospective evaluation of CRT effects on anorectal function using anal manometry was published by Ammann et al.,who compared a CRT treated group with a group who underwent surgery alone.[67] Patients undergoing preoperative CRT showed a significantly decreased mean resting pressure, resting vector vol-

ume and maximal tolerable volume one year postoperatively while these parameters were unaffected in patients treated with surgery alone. Interestingly, low and rectoanal anastomoses resulted in better anorectal function than a high anastomosis in CRT treated patients. Preliminary functional and quality of life (QoL) data from the EORTC 22921 trial were recently presented.[68] This four arm trial randomized patients to preoperative RT versus CRT and to adjuvant chemotherapy versus no adjuvant CT. Compared to RT alone, preoperative CRT was associated with a significantly worse global QoL and anorectal function in patients who underwent sphincter saving surgery. These preliminary data suggest that the addition of chemotherapy signifies an increased burden to the patients in terms of QoL and anorectal function and this should be taken into account when discussing treatment options.

3.4.9 Future Perspectives

Since the improvement in local control with preoperative CRT has until now not been reflected in a survival advantage, one of the areas of future improvement will be the addition of more effective systemic therapy to eradicate microscopic systemic disease. Clinical trials using a combination of chemotherapy with targeted therapy as a component of CRT are underway.[69] Therapeutic decisions and prognostic assessment will increasingly be based on genomic and molecular profiling of the tumour in addition to standard imaging methods. Improvements in radiation technique such as conformal and intensity modulated RT will allow to limit toxicity by better defining the target volume

while limiting exposure of normal tissue.[70,71]

3.4.10 Conclusion

Preoperative CMT is superior to adjuvant CMT and, compared to preoperative RT alone, enhances tumour response while acute toxicity increases. Preoperative CMT could have a role in patients with possibly invaded resection margins or low lying cancers, although in the randomized trials tumour downstaging did not always translate into an increase in sphincter preservation. Observation of a clinical complete response to CMT does not obviate the need for surgery. Preliminary data from the EORTC 22921 randomized study suggest that the addition of chemotherapy to preoperative RT adversely affects both overall QoL and anorectal function. The final results of ongoing randomized trials will more accurately establish the role of preoperative CMT in resectable rectal cancer patients.

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Chapter 4

Neoadjuvant Chemoradiation versus Hyperfractionated Accelerated Radiotherapy (HART) in Locally Advanced Rectal Cancer

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4.1 Abstract

Background: neoadjuvant therapy is increasingly used in resectable locally advanced rectal cancer. The exact role of the addition of chemotherapy (CT) is not established. We compared neoadjuvant therapy using chemoradiation (CRT) with hyperfractionated accelerated radiotherapy (HART).
Methods: Clinical, pathological and survival data were obtained from patients with resectable stage II or III rectal cancer within 7 cm from the anal verge. A group of 50 patients was treated with a preoperative dose of 41.6 Gy of radiotherapy (RT) in 2 daily fractions of 1.6 Gy over 13 days immediately followed by surgery (HART). A second group of 96 patients received 45 Gy of conventionally fractionated RT in 25 daily fractions of 1.8 Gy combined with infusional 5-FU based chemotherapy followed by surgery within 4-6 weeks (CRT). Both groups were compared in terms of morbidity, pathological downstaging, local recurrence, and survival.
Results: Both groups were comparable in terms of preoperative clinicopathological variables. The mean distance from the anal verge was 5.8 cm (HART) versus 4.9 cm (CRT). Sphincter preservation was possible in 74% (HART) versus 83.5% (CRT) of patients (p=0.013). The clinical anastomotic leak rate was 2% (HART) versus 2.2% (CRT). pCR was observed in 4% (HART) versus 18% (CRT) of the resected specimens (p=0.002). A pelvic recurrence developed in 6% (HART) versus 4.4% (CRT) of patients (p=0.98). Overall 5 year survival was 58% (HART) versus 66% (CRT), p=0.19 while disease free 5 year survival was 51% (HART) versus 62% (CRT), p=0.037.

Conclusions: Compared with preoperative HART followed by immediate surgery, preoperative CRT followed by a 6 weeks waiting period enhances pathological response and increases sphincter preservation rate. This could be explained by the addition of chemotherapy or the longer interval between neoadjuvant therapy and surgery. No statistically significant difference was observed in local control or overall survival.

4.2 Introduction

The annual incidence of fatal cases of colorectal cancer exceeds 200.000 in the European Union alone. The mainstay of therapy in locally advanced rectal cancer remains surgery with negative margins, including the circumferential resection margin (CRM). Historically, surgery for rectal cancer has been associated with locally recurrent disease in up to one in four patients.[1]
Data from a recent systematic review suggest that preoperative radiotherapy (RT) lowers local recurrence rates provided a biologically equivalent dose of at least 30 Gy is administered.[2] On the other hand, attention to surgical technique with precise sharp dissection of the mesorectal plane and total mesorectal excision (TME) in lower third cancers significantly improved local control in a number of expert series.[3-6] The question whether preoperative RT remains effective when optimal surgical technique is systematically implemented was convincingly answered by the results of the Dutch Rectal Cancer Trial.[7] After a nationwide surgical training program, preoperative RT further reduced local recurrence rate (2.4% after RT+TME versus 5.3%

in the TME alone group, $p<0.001$).

For patients with bulky tumors, possibly invaded lateral resection margins or tumors close to the sphincter apparatus, several approaches to intensify preoperative RT have been employed. The addition of chemotherapy to preoperative RT builds on the favorable results obtained with postoperative chemoradiation (CRT) and preoperative CRT for irresectable disease.[8] Several phase II trials with preoperative CRT in resectable rectal cancer have shown a promising pathological complete response (pCR) rate and a high rate of sphincter preservation.[9,10]

The aim of hyperfractionated regimens is to separate early and late radiation effects aiming to improve local control while limiting late tissue toxicity.[11] In head and neck cancer, a randomized trial by the European Organization for the Research and Treatment of Cancer (EORTC) demonstrated a significantly better local control after hyperfractionated RT compared to a conventionally fractionated regimen.[12] In colorectal cancer, cell kinetic studies using biomarkers have demonstrated rapid proliferation of clonogens, with a small potential doubling time (Tpot) which could cause local tumor recurrence.[13-15] Moreover, because of potential rapid regrowth of subclinical tumor deposits during RT, limiting total treatment time is important to achieve a high probability of local control.[16] Theoretically, therefore, rectal tumors would benefit from hyperfractionated accelerated radiotherapy (HART).

We retrospectively compared two neoadjuvant therapy regimens in patients with resectable locally advanced rectal cancer. Group 1 was treated with HART immediately followed by surgery. Group 2 received neoadjuvant 5-FU based CRT followed by

surgery after a 4-6 weeks interval.

4.3 Methods and Materials

4.3.1 Patient Selection

All patients with resectable rectal cancer stage cT3-4 N0 or cT1-4 with nodal disease were offered neoadjuvant therapy. Patients with possibly resectable lung or liver metastases were also included. From 1994 until 7/2000, all patients received preoperative HART. After this period, given the results from various phase II trials in both resectable and irresectable rectal cancer, neoadjuvant chemoradiation was introduced. Mean follow up time for both groups was therefore different. Since most local recurrences following rectal cancer surgery occur within two years postoperatively and no major changes were applied in surgical technique, we considered a comparison of both protocols appropriate when looking at downstaging and local control as endpoints.

4.3.2 Preoperative Workup

Preoperative clinical staging included clinical assessment, liver ultrasound or CT scan, chest X-ray or CT scan, full blood analysis including CEA and colonoscopy with biopsies. Routine use of magnetic resonance imaging before and after neoadjuvant therapy was introduced in 2002 and performed in 42 patients (30%). Endoscopic ultrasound (EUS) of the tumor was performed in

52% of the patients.

4.3.3 Radiotherapy

All patients were treated on a 25 MV Elekta linear accelerator. The treatment was delivered in prone position with a three-field technique (one posterior and two opposite lateral fields) on the pelvis. The upper border was set at the L5-S1 interspace. If an abdominoperineal resection was to be performed, the perineum was included in the fields. The anterior border of the lateral fields was set just posterior of the symphysis, the posterior border included the sacrum. Adequate blocking was used to exclude excessive amounts of small intestine. The dose was prescribed at the isocentre. A combination of wedged and open fields was used, depending on which resulted in the most optimal dose distribution, with a homogeneity within 5% of the prescribed dose, according to the ICRU rules. The patients in the HART group were treated twice daily, five days a week, with an interval of at least 6 hours. The dose per fraction was 1.6 Gy. Twenty-six fractions were delivered, resulting in a cumulative dose of 41.6 Gy. The patients in CRT group were treated once daily, five days a week, with a dose of 1.8 Gy. Twenty-five fractions were delivered, resulting in a cumulative dose of 45 Gy.

4.3.4 Chemoradiation

In 85 (93%) patients, chemotherapy consisted of bolus 5-fluorouracil (325 mg/m2) and folinic acid (10 mg/m2) given during day 1-5 and 29-33 of RT. In 6 (7%) patients, concomitant chemother-

apy consisted of oxaliplatin (50 mg/m2) weekly for 5 weeks and capecitabine (825 mg/m2) BID 5 days per week during 5 weeks, according to the CORE protocol (first results presented at ECCO 2005, Paris [17]).

4.3.5 Surgery

All patients underwent nerve sparing total mesorectal excision (TME). The decision to perform a sphincter sparing procedure was made peroperatively, and not before initiation of neoadjuvant therapy. Technical details included division of the inferior mesenteric artery and vein at 1 cm from its origin, routine mobilization of the splenic flexure and washout of the rectal stump with an iodine solution prior to completion of the anastomosis. Creation of a temporary loop ileostomy was performed in selected cases as judged necessary by the operating surgeon.
The criteria for sphincter preservation were: acceptable sphincter function and absence of direct invasion of the sphincter apparatus. These criteria remained unchanged throughout the treatment period.

4.3.6 Follow Up

During treatment, patients were seen weekly and acute toxicity was scored according to the WHO scale. Late toxicity was evaluated at least 12 months after surgery. Node positive (stage III) patients were proposed six cycles of adjuvant chemotherapy.

4.3.7 Statistical Analysis

Data are expressed as mean (standard error), unless indicated otherwise. Differences between means of continuous variables were analyzed with the 2-tailed t-test or, when a non-normal data distribution was observed, with the Mann Whitney U test. Differences between fractions were analyzed with the Chi squared or Fisher's exact test where appropriate. Actuarial survival curves were generated with the Kaplan Meier method and compared using the log rank test. Statistical significance was assumed at an alpha value <0.05. All calculations were performed with SPSS 12.0 for Windows.

4.4 Results

4.4.1 Clinicopathological Variables

As illustrated in Table 4.1, no significant differences were present between both groups regarding demographic variables, clinical tumor stage, or distance between tumor and the anal verge. One third of patients in both groups had a tumor within 3 cm from the anal verge. More than half of the patients had clinically node positive disease, and hepatic metastases deemed resectable were present in approximately 10% of patients in both groups.

4.4.2 Neoadjuvant therapy acute toxicity

Acute gastrointestinal, urogenital and hematological toxicity was more pronounced in the group of patients receiving CRT,

Variable	Group 1 (HART) N=50	Group 2 (CRT) N=91	p
Age (years)	60.6 (1.7)	61.8 (1.1)	0.52
Male patients (%)	70	77	0.11
BMI (kg/m2)	23	26.2 (2.1)	0.41
Preoperative CEA (ng/ml)	11.8 (4.1)	16.9 (5.8)	0.55
Distance from anal verge (cm)	5.8 (0.6)	4.9 (0.4)	0.23
Tumor < 3 cm from anal verge (%)	34	33	0.99
Tumor length (cm)	5 (0.7)	3.8 (0.5)	0.16
cT stage			0.40
1	0	1	
2	2	4	
3	26	67	
4	8	9	
cN stage			0.89
0	13	27	
1	26	45	
2	2	3	
cM stage			0.99
M0	45	82	
M1	5	9	

Table 4.1: Comparison of preoperative clinicopathological variables. Data represent mean (standard error) unless indicated otherwise. BMI, body mass index; CEA, carcinoembryonic antigen; HART, hyperfractionated accelerated radiotherapy; CRT, chemoradiation.

Site	Group 1 (HART) N=50	Group 2 (CRT) N=91
Gastrointestinal		
Grade 1	14	32
Grade 2	2	8
Grade 3	-	-
Renal, Bladder		
Grade 1	2	10
Grade 2	-	1
Grade 3	-	-
Cutaneous		
Grade 1	5	12
Grade 2	2	5
Grade 3	-	1
Hematological		
Grade 1	-	2
Grade 2	-	1
Grade 3	-	-

Table 4.2: Comparison of WHO acute toxicity episodes. HART, hyperfractionated accelerated radiotherapy; CRT, chemoradiation.

whereas cutaneous toxicity was comparable (Table 4.2). Grade three toxicity was seen in only one patient, who received CRT and developed a severe skin reaction. No grade four toxicity was observed.

4.4.3 Details and Outcome of Surgery

Details of surgery are provided in Table 4.3. Surgery was performed after a mean time interval of 3 days (HART) versus 6 weeks (CRT). Significantly more sphincter preserving procedures were performed in patients who received CRT. No difference was observed in clinical anastomotic leak rate. Both early postoperative overall morbidity and late radiation induced toxicity, however, were significantly more pronounced in group 1 (HART) patients. In the HART group, 8 patients (16%) developed late radiation sequelae. Five patients (10%) needed surgery for severe late radiation induced bowel damage (radiorectitis 2, radioenteritis 1, rectovaginal fistula 2). Another three patients in the HART group developed radiorectitis but were managed medically. The median time interval between primary and reoperative surgery was 15 months (range 2-34). In the CRT group, two patients (2.2%) developed radioenteritis but did not undergo reoperation.

4.4.4 Pathology data

Pathological details and downstaging rates are illustrated in Table 4.4. Both tumor and node downstaging were significantly more pronounced in group 2 (CRT). The pathological complete response rate was significantly higher in patients who received CRT. Downsizing of the resected tumor was observed in both groups compared to the preoperative tumor size. In the CRT group, however, tumors were significantly smaller after neoadjuvant therapy compared to the HART group. Mucinous differ-

Variable	Group 1 (HART) N=50	Group 2 (CRT) N=91	p
Interval NT-surgery (d)	3.4 (0.7)	42.4 (1.5)	<0.001
Sphincter saving (%)	74	83.5	0.013
Defunctioning ostomy (%)	45.9	86.9	<0.0001
Anesthesia time (min)	281.7 (7)	283.5 (7.5)	0.88
Gastric drainage (d)	4.4 (0.3)	3.7 (0.3)	0.07
Hospital stay (d)	20.5 (1.9)	15 (0.7)	0.002
In hospital mortality (%)	0	2	0.76
In hospital morbidity (%)	68	46.2	0.021
Anastomotic leak rate (%)	2	2.2	0.6
Interval surgery-ostomy closure (d)	95 (17.4)	81.6 (8.4)	0.5
Late radiation toxicity (%)	16	2.2	0.004
Adjuvant chemotherapy given (%)	38	39.6	0.9

Table 4.3: Details and results of the surgical procedure. Data represent mean (standard error) unless indicated otherwise. NT, Neoadjuvant Therapy; HART, hyperfractionated accelerated radiotherapy; CRT,chemoradiation.

Variable	Group 1 (HART) N=50	Group 2 (CRT) N=91	p
Tumor size (mm)	34.4 (2.6)	22.8 (1.2)	<0.001
Mucinous diff.	9 (18)	16 (18)	0.99
pT downstaging	15 (30)	45 (51)	0.02
pN downstaging	9 (18)	35 (38)	0.012
pCR	2 (4)	16 (18)	0.002

Table 4.4: Comparison of pathological details and tumor (T) and node (N) downstaging. pCR, pathological complete response (pT0N0). Data represent mean (standard error) unless indicated otherwise. HART, hyperfractionated accelerated radiotherapy; CRT, chemoradiation. Values between brackets are percentages.

entiation was observed in 18% of patients in both groups. Although the percentage of patients in both groups receiving adjuvant chemotherapy was similar, all patients in group 1 (HART) received a 5-fluorouracil alone regimen whereas in group 2 (CRT), the regimen contained oxaliplatin or irinotecan in 42% of patients.

4.4.5 Local Control and Survival

Mean follow up time was 67 months in group 1 (HART) and 28 months in group 2 (CRT) (p<0.001). No patients were lost to follow up. Overall local recurrence rate was 6% in group 1 (HART) and 4.4% in group 2 (CRT) (p=0.98). In group 1,

all three local recurrences were isolated. Two of these patients had a pT4 tumor and developed a presacral recurrence, while in the third patient a lymph node recurrence developed along the left iliac artery. In group 2, three of the local recurrences were seen in patients with systemic disease while isolated local recurrence was present in only one patient who presented with pT4 disease with bilateral ureteral obstruction. The isolated local recurrence rate was therefore 3% in group 1 versus 1.1% in group 2 (p=0.13). As far as distant failure is concerned, 24% of pre-treatment clinically M0 patients developed metastases in group 1 (HART) and 11% in group 2 (CRT), p=0.02.
Survival data are illustrated in figures 4.1 and 4.2. Overall 5-year survival was 58% in group 1 and 66% in group 2 (p=0.19). Disease free 5-year survival was 51% in group 1 and 62% in group 2 (p=0.037).

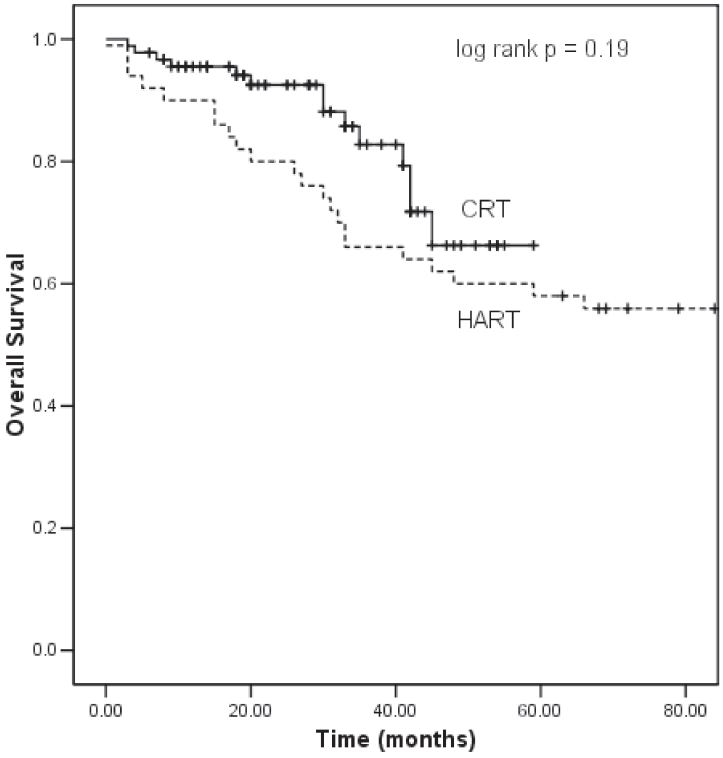

Figure 4.1: Comparison of overall survival. HART, hyperfractionated accelerated radiotherapy; CRT, chemoradiation.

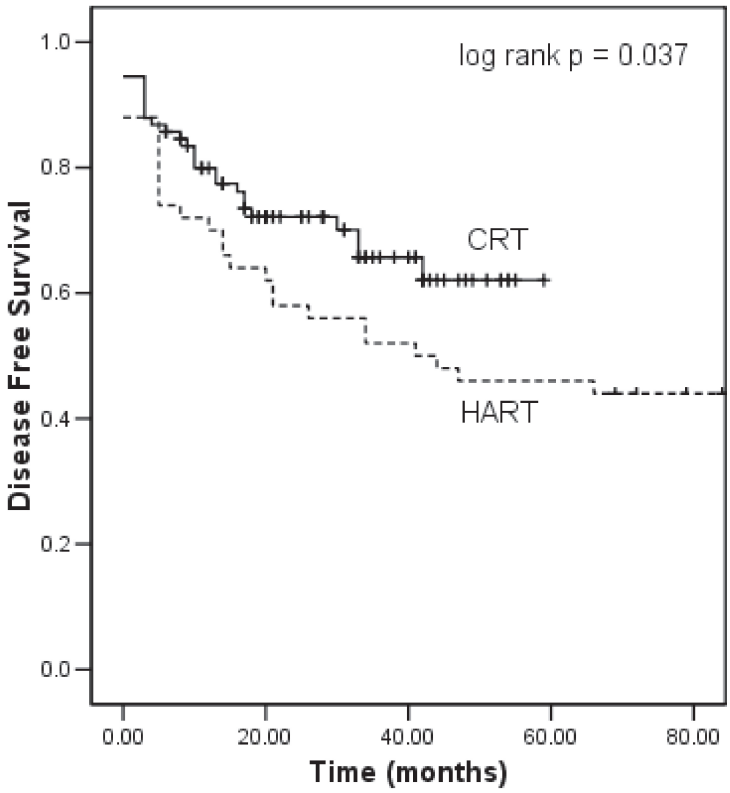

Figure 4.2: Comparison of disease free survival. HART, hyperfractionated accelerated radiotherapy; CRT, chemoradiation.

4.5 Discussion

A multimodal approach has become the standard of care in locally advanced resectable rectal cancer. The addition of chemotherapy to RT is firmly grounded in the principles of enhanced tumor response and spatial cooperation (local versus systemic activity).[18] Several theoretical considerations favor preoperative CRT over postoperative CRT, and this was clinically demonstrated in the recent German rectal cancer trial.[19] We retrospectively compared neoadjuvant HART immediately followed by surgery with neoadjuvant CRT with surgery performed after a 6 weeks period. Several non-randomized and randomized studies have compared RT alone with CRT in the preoperative therapy of rectal cancer. From the EORTC 22921 and the French FFCD 9203 trials, only preliminary data are available. Taken together, the results of these studies indicate that the addition of CT significantly improves tumor response, although acute toxicity is usually worse. Interestingly, the increase in pathological downstaging was translated into enhanced sphincter preservation in only 1 of the 4 randomized studies. This finding may be explained by the reluctance of surgeons to perform a low anastomosis with tissue that was cancer invaded before initiation of CRT. Moreover, the observed increase in tumor response did not improve disease free or overall survival. Our results confirm an increased tumor response with CRT, with significantly increased downstaging of both T and N status. Probably, the difference in downsizing and downstaging between both groups is mainly due to the different waiting period between the completion of RT and surgery. In our experience, this was translated

into more sphincter preserving procedures, although we cannot exclude the influence of other variables such as refinements in surgical technique allowing more very low anastomoses to be performed. In keeping with others, we found that preoperative RT in either schedule increases the incidence of mucinous carcinomas.[20] The anastomotic leak rate was not affected by neoadjuvant therapy, confirming experimental data indicating that neither RT nor CRT affect colonic anastomotic healing provided only one limb of the anastomosis is irradiated.[21,22] The low clinical anastomotic leak rate in the present series is partly explained by the liberal use of a deviating ileostomy. Although presence of an ileostomy temporarily adversely affects quality of life,[23] it has been shown to reduce the incidence of clinical leakage and to mitigate the consequences of a leak.[24]
On the other hand, early (30 day) postoperative overall morbidity (major and minor) was significantly more pronounced in patients treated with HART, reflected by a significantly longer hospital stay. It is possible, however, that a less liberal use of a diverting ileostomy in HART patients is confounding the difference in 30 day morbidity rate. The rationale for HART in rectal cancer is based on the observation of the relatively early occurrence of locally recurrent disease in most patients. At the origin of a local recurrence are rapidly proliferating clonogens with a small potential doubling time (Tpot).[14-16] Limiting the total treatment time by accelerating RT and reducing the delay with surgery could therefore enhance the effect on local recurrence after rectal cancer surgery. Clinical experience with preoperative HART in rectal cancer is limited. Coucke et al. reported on two phase I trials in 20 patients treated with postoperative 41.6

Gy of HART and 23 patients treated with 41.6 Gy of preoperative HART.[25] Acute toxicity after preoperative HART was acceptable in this report, and small bowel toxicity was significantly lower compared to postoperative HART. There was only one case of late bowel toxicity in the group treated preoperatively. The same group recently reported a phase I study combining preoperative 41.6 Gy of HART with concomitant CPT-11 in locally advanced rectal cancer.[26] Major complications after surgery were seen in 25% of patients with a considerable anastomotic leak rate of 22%.

Hyperfractionation is aimed at limiting late tissue toxicity while achieving an identical or enhanced local control. In head and neck cancer, a randomized trial comparing a hyperfractionated (twice daily 1.15 Gy) with a conventional (daily 2 Gy) regimen has shown a significant increase in local control (59% versus 40% at 5 years) with no increase in late toxicity.[12] We could not demonstrate the late toxicity sparing of HART and, on the contrary, found a significant increase in late radiation sequelae with 10% of patients needing further surgery as a direct consequence of radiation induced bowel damage. Certainly, this conclusion has to be interpreted with caution and may even be premature, given the significant difference in follow-up time between both groups. Overall and isolated local recurrence rates were low with both treatment modalities and in keeping with recent clinical literature. More specifically, no influence of limiting total treatment time was noted on the incidence of local recurrence. One of the important questions in low-lying rectal cancer is how the time interval between preoperative therapy and surgery, tumor downstaging and sphincter preservation rate are related.

In the Polish randomized study comparing preoperative CRT with 5x5 Gy of preoperative RT, the observed increase in tumor response was not translated into enhanced sphincter preservation.[27] This was explained by the fact that the surgeon's decision to perform a sphincter saving procedure was based on the pre-treatment tumor volume. We chose to rely on the post-treatment clinical stage, with acceptance of a peroperative free distal resection margin of >5 mm when confirmed by frozen section analysis. This policy resulted in a significantly higher percentage of sphincter saving procedures in CRT patients (83.5% versus 74%, p=0.013).

Survival and recurrence data are to be interpreted with caution as the mean follow up time between both groups is significantly different. Moreover, routine preoperative MRI to define the circumferential margin was performed only in the CRT group which could lead to stage migration. Since most patients were followed for more than 2 years, we considered it useful to provide the disease free and overall actuarial survival data. Overall 5-year survival was not different between the two treatment modalities. Disease free 5-year survival, however, was better in the CRT group (62% versus 51%, p=0.037). This is explained by a significantly lower occurrence of systemic failure after CRT (11% versus 24%, p=0.02) confirming the effectiveness of the systemic component of this neoadjuvant regimen in patients who may already have micrometastatic disease at presentation. However, differences in the adjuvant therapy regimen in stage III patients could partly account for the observed difference in DFS and distant metastasis rate.

In conclusion, preoperative CRT increases tumor downsizing

and downstaging compared to preoperative HART. This translates into a higher sphincter preservation rate at the expense of a moderately increased acute toxicity. Postoperative both early and late complications are significantly more pronounced after HART. Both regimens when combined with precise surgery result in excellent local control. Systemic relapse was more common and disease free survival worse after HART, but overall survival was not different.

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Chapter 5

Noninvasive Monitoring of Radiotherapy Induced Microvascular Changes using Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI) in a Colorectal Tumor Model

W Ceelen, P Smeets, W Backes, N Van Damme, T Boterberg, P Demetter, I Bouckenooghe, M De Visschere, M Peeters, P Pattyn. Int J Radiat Oncol Biol Phys 2006; 64: 1188-1196

5.1 Abstract

Purpose: Prediction of response to radiotherapy (RT) in colorectal cancer is highly relevant for both surgical management and prognosis in these patients. In a rat colorectal cancer model, we studied the use of dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) with a new macromolecular rapid clearance MR contrast agent (P792, gadomelitol) (i) to visualize tumor vascular leakage as a surrogate marker for angiogenesis, (ii) to evaluate the effects of RT on this parameter, and (iii) to compare noninvasive imaging with invasive pO2 measurement, vascular endothelial growth factor (VEGF) expression, microvascular density (MVD), and pimonidazole hypoxia staining.

Methods and Materials: male WAG/Rij rats were injected with 2x106 CC531 cells in the hind leg. Once the tumor reached a diameter of 8 mm, fast DCE-MRI was performed before and 5 days after 5x5 Gy of external RT. The DCE-MRI data were used to generate parametric tissue maps of the endothelial transfer constant (Ktrans) according to the Tofts-Kermode pharmacokinetic two-compartment model. Separate region of interest (ROI) analyses were performed on the entire tumor (T), peripheral tumor rim (P), tumor core (C), and normal muscle (M). Invasive fiber optic tissue pO2 histogram mapping was performed in each tumor core and rim before and after RT using a stepwise micromanipulator technique. Finally, MVD counts, VEGF expression and pimonidazole hypoxia staining were performed after RT in excised tumors and compared with a group of untreated tumor bearing rats.

Results: the biophysical properties of P792 allowed excellent discrimination between tumor and normal tissue. Mean (x1000/min) value changes measured over all pixels in a ROI were as follows: T: 14.6 vs 3.8 ($p<0.001$); P: 26.3 vs 4.5 ($p<0.001$); C: 5.4 vs 1.8 ($p<0.001$), and M: 3.3 vs 2.1 ($p=0.12$) before and after RT respectively. Mean pO2 was P: 6.8 mm Hg before RT vs 7.7 mm Hg after RT ($p<0.001$) and C: 3.5 mm Hg before RT vs 4.4 mm Hg after RT ($p<0.001$). Mean MVD in the tumor rim was 10.4 in the RT treated group vs 16.9 in the control group ($p=0.061$); MVD in the tumor core was not significantly different. VEGF expression was significantly higher in RT treated rats, but pimonidazole hypoxia score was not significantly different. After RT, no significant correlation was found between DCE-MRI parameters and histological parameters. An inverse correlation was seen after RT between pO2 and Ktrans ($r=-0.57$, $p=0.08$) and between pO2 and Ve ($r=-0.65$, $p=0.04$).
Conclusions: DCE-MRI with P792 allows non-invasive imaging and quantification of microvascular changes in this colorectal cancer model. Administration of short term RT significantly reduces neovascular leakage and enhances tissue oxygenation and VEGF expression. After RT, DCE-MRI parameters are related to tumor pO2, but not to MVD or VEGF expression.

5.2 Introduction

Colorectal cancer remains one of the leading causes of cancer death in the Western world.[1] Due to the specific anatomy and biology of low rectal cancer, surgical resection alone his-

torically has been associated with a high incidence of locally recurrent disease. Neoadjuvant combined modality treatment has been shown to decrease local recurrence rates even with the use of optimal surgical technique (total mesorectal excision).[2,3] Potential disadvantages of neoadjuvant chemoradiation include overtreatment of overstaged disease, treatment related toxicity and long term effects on sphincter function.[4] There is therefore a need for noninvasive imaging techniques that allow clinicians to predict and monitor tumor response to neoadjuvant therapy. Magnetic resonance imaging (MRI) recently has evolved as one of the most promising imaging modalities in the diagnosis and staging of rectal cancer.[5] Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) using paramagnetic contrast agent administration allows one to analyse tumor specific enhancement patterns that are governed by physiological properties such as microvascular flow, endothelial permeability and size of the extracellular extravascular space (EES).[6] Quantitative analysis of the enhancement signal using a pharmacokinetic model can generate estimates for parameters such as the endothelial transfer coefficient Ktrans.[7] The MRI contrast agents currently in clinical use have a low (< 1 kDa) molecular weight, diffuse readily across the endothelial barrier of both normal and neoplastic tissues and therefore are much less suited to characterize hyperpermeable neoplastic vessels.[8] Macromolecular weight contrast media take advantage of the selective hyperpermeability of neoplastic vessels to macromolecules and have been used successfully to monitor angiogenesis and the effects of anti-angiogenesis agents in preclinical studies.[9] For macromolecular agents, microvascular leakage determined by Ktrans

is relatively flow independent and therefore estimation of leakage changes as a surrogate marker of angiogenesis is possible without exact measurement of capillary flow.[10]
We studied the changes in neovascular leakage of an experimental colorectal cancer during fractionated radiotherapy (RT) using DCE-MRI with P792, a new macromolecular MRI contrast agent (CA) currently evaluated in phase II clinical trials. Non-invasive imaging was compared with invasive tissue pO2 measurement, microvessel counts, vascular endothelial growth factor (VEGF) expression, and pimonidazole hypoxia staining.

5.3 Methods and Materials

The experimental protocol was reviewed and approved by the Animal Ethical Committee of the Ghent University, Belgium.

5.3.1 Animals and Tumor Model

A group of 11 male Wag/Rij rats (Harlan, Horst, The Netherlands) was studied longitudinally with DCE-MRI and invasive oxygenation measurements performed both before and 5 days after completion of RT. For histology and immunohistochemistry, this group was compared with 9 untreated control rats bearing tumors of similar size.
The CC531 cell line is a 1,2-dimethylhydrazine-induced, moderately differentiated and weakly immunogenic colon adenocarcinoma, syngeneic with WAG/Rij rats. This cell line is well studied and has been proven to provide a tumor-host model

similar to human colorectal carcinogenesis.[11] Cells were grown as a stationary cell line in plastic culture flasks in RPMI 1640 medium, buffered with HEPES (20 mM) (Invitrogen Corporation, Gibco, Ghent, Belgium) additionally supplemented with 10% fetal calf serum, 4 mM L-glutamine, 50 U/ml penicillin and 50 g/ml streptomycin at 37 C in a humidified atmosphere with 5% CO2 in air. The cells were transferred at 95% confluency. Two million cells suspended in 0.2 ml were injected subcutaneously in the upper hind leg. Tumors reached a size of 0.5-1 cm after a period of 4 weeks. Once tumor growth of minimally 8 mm diameter was observed, a jugular vein catheter was inserted and tunneled to the interscapular region. In order to maintain catheter delivery function between the first and second MRI, continuous infusion at 0.5 ml saline per hour was administered with a cage mounted swivel and flexible metal tether system (Uno BV, Didam, The Netherlands) allowing the animal full mobility. To enable histological assessment, animals were sacrificed by anesthetic overdose after the last in vivo measurements.

5.3.2 Radiotherapy

Rats were not sedated and the tumor bearing hind leg was immobilized using a plexiglass holder, as described previously.[12,13] Briefly, rats were placed in a purpose-built plexiglass holder in prone position. The hind legs were pulled through an opening in the holder and immobilized with strings. Before each fraction, a radiation field was simulated encompassing the tumor with a margin of 1.5 cm. The photon irradiation was performed with a 5 MV linear accelerator (Elekta, Crawley, UK). Five fractions of

5 Gy (total dose 25 Gy) were delivered on five consecutive days. Since the tumors were inoculated subcutaneously, they were covered with tissue-equivalent silicone bolus of 1 cm to prevent the build-up effect under the skin. One single direct field at a fixed source-skin distance of 100 cm was used. The dose was calculated to the midpoint of the tumors according to their volume in each individual animal, as obtained during simulation. DCE-MRI and oxygenation measurements were performed before and 5 days after the completion of RT.

5.3.3 Magnetic Resonance Imaging

T1 weighted DCE-MRI was performed on a clinical Siemens Magnetom Symphony 1.5 Tesla scanner (Siemens AG, Erlangen, Germany). Animals were sedated with 0.2-0.4 ml of medetomidine (Domitor, Novartis Animal Health, Basel, Switzerland). Imaging comprised a single axial slice that was positioned through both upper limbs and the center of the tumor. Prior to the contrast series, T1 zero time maps were constructed from two spin echo sequences with different repetition times (TR 1000 ms and 318 ms, respectively). Details of this sequence were as follows: slice thickness 3 mm, FOV 140x88, matrix size 256x160, TE 20 ms, and flip angle 90 degrees. Dynamic imaging was performed with a 4 antenna wrist coil (diameter 10 cm) using an IR-TurboFLASH sequence. Details of the pulse sequence were as follows: temporal resolution 1.1s, FOV 140x88, matrix size 256x160, slice thickness 5 mm, TE 4.08 ms, TI 560 ms, and flip angle: 12 degrees. A bolus injection of 0.3-0.4 ml of P792 was manually injected as fast as possible (approximately 1ml/s)

through the central venous line after the fourth scan. A total of 500 images were obtained for a total scan time of 550 seconds.

5.3.4 Analysis of MR Images

Postprocessing was performed using the research mode of a commercially available software tool (MIStar, Apollo Medical Imaging, Melbourne, Australia).
The maximum cross sectional area (in cm2) of each tumor was recorded before and after RT.
Both a qualitative description of the tissue enhancement curve and a 2 compartment pharmacokinetic approach according to Tofts and Kermode were implemented.[7] In each tumor, regions of interest (ROI) were drawn encompassing the following regions: the entire tumor (1), the angiogenic tumor rim defined as the outer 2-3 voxel wide circumference (2), the central necrotic area (3) and muscle tissue in the contralateral non irradiated hind leg (4).
In each region, the area under the enhancement curve (AUC) was calculated until 550 seconds after contrast arrival.
Pharmacokinetic modeling was based on a 2 compartment model consisting of a vascular space and an EES fraction which is entered by the CA leaking through the microvascular wall. Derived from a first order differential equation describing contrast agent flux driven by Fick's law of diffusion, the concentration of CA in the tissue (Ct in mM) is described by the following equation:

$$C_t = K^{trans} \int_0^t C_p(t') e^{\frac{-K^{trans}}{v_e}(t-t')} dt'$$

where Ktrans (min-1) denotes the endothelial transfer rate, Ve the fraction of the interstitial space (dimensionless) entered by the CA and Cp the plasma concentration (in mM). In each tumor both before and after RT, the pixel with the most representative arterial input function (AIF) was manually selected from the femoral artery of the tumor bearing leg or the opposite leg to provide Cp. Mean tumor tissue and arterial T1 zero (before contrast injection) values were calculated from the T1 zero maps. These data together with the selected AIF were used as the input for a curve fitting routine resulting in parametric maps of Ktrans and Ve. The influence of inflow effects on leakage measurements was minimized by using a deconvolution method in the model to separate the inflow component from the tissue concentration Ct.[7]
Quantitative values for all pixels in the 4 different regions of interest described above were exported to a spreadsheet for further analysis.

5.3.5 MR Conrast Agent

Dynamic contrast studies were performed with P792 (gadomelitol, Vistarem, Guerbet, Roissy, France), a new monogadolinated rapid clearance MRI blood-pool agent which is cleared by renal elimination. The molecular weight of the compound is 6.47 kDa, but the mean diameter of P792 is 50.5 Angstrom and the T1 relaxivity of this agent is 29 M-1 ms-1 at 60 MHz.[14] Apparent

hydrodynamic volume of P792 is 125 times greater than that of Gd-DOTA (gadoterate meglumine, Dotarem) and as a result of this high molecular volume, P792 is characterized by a limited diffusion across normal endothelium and is therefore ideally suited to study hyperpermeable neoplastic vessels.[14] Experimentally, P792 has been used to study permeability effects of anti-angiogenesis therapy in a prostate cancer model.[15]

5.3.6 Tissue Oxygenation Measurements

Tissue oxygenation was measured in both the tumor core and periphery with a fiberoptic probe based on fluorescence quenching (OxyLite, Oxford Optronix, Oxford, UK).[16,17] A precalibrated fiberoptic probe (diameter 280 μm) was inserted 5 mm deep into the tumor using a Seldinger technique. This involved insertion of a catheter with needle assembly into the tissue; the needle was then withdrawn leaving the catheter in place through which the probe was inserted in such a way that the probe's tip was exposed to the surrounding tissue. The probe was then withdrawn in 40 steps of 100 μm each over a total distance of 4 mm using a micromanipulator (model MN151, Narishige International Ltd, London, UK).

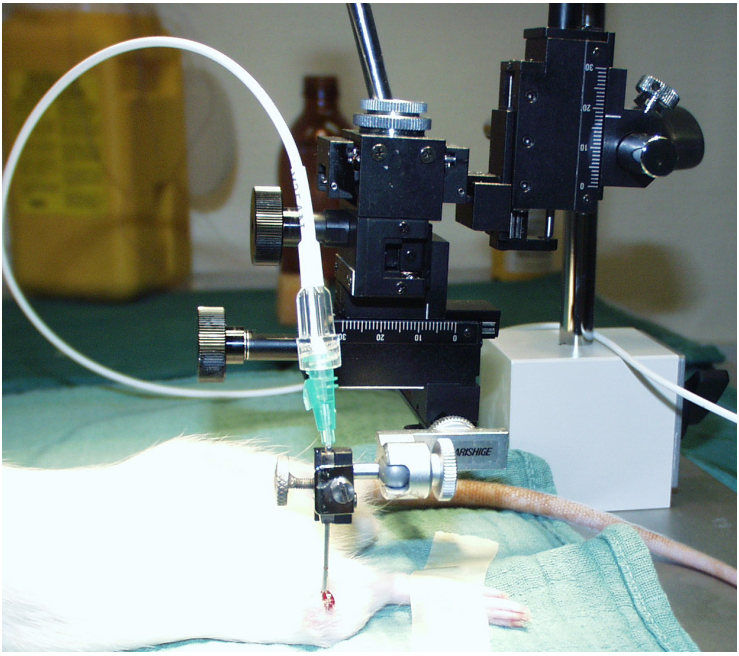

Figure 5.1: Measurement of tissue pO2 by a fiberoptic probe inserted in the tissue by a Seldinger technique. The probe is withdrawn in steps of 0.1 mm by a micromanipulator.

After each micromanipulator movement, measurements were started as soon as a stable reading was obtained. Tissue pO2 was sampled every 2 seconds. Histograms were constructed based on the pO2 (expressed in mm Hg) readings over the cen-

tral one mm (core) and outer one mm (periphery) trajectory. Both before and after RT, the hypoxic fraction (defined as percentage of pO2 measurements with a value of <5 mm Hg) was determined.

5.3.7 Immunohistochemistry

Immunohistochemistry was performed on the experimental group and on 9 untreated control rats bearing tumors of similar size. From each tumor, half of the tissue was snap frozen in liquid nitrogen; the other part was fixed in 4% formalin and embedded in paraffin wax in the conventional manner. Microvascular density (MVD) was determined with a method modified after Weidner et al.[18] After incubating 5 micron frozen slices with anti-CD31 antibodies (mouse anti rat CD31, clone number TLD-3A12, Serotec, Oxford, UK), the entire tumor section was scanned at low power (objective, 40 X) to identify 'hot spots', which are the areas of highest neovascularization. Individual microvessels were then counted under higher power (objective, 400X) to obtain a vessel count in a defined area, and the average vessel count in 3 hot spots was taken as the MVD.
For hypoxia staining, rats were injected with 60 mg/kg iv pimonidazole (Hydroxyprobe Kit, Biognost, Heule, Belgium) 30 minutes before sacrifice. Paraffin embedded slides were incubated with anti-pimonidazole antibodies and the resulting cytoplasmatic staining was expressed semiquantitatively. Membrane staining was not observed. Immunoassaying was visualized using 3-amino-9-ethylcarbazole (Dako, Glostrup, Denmark).
VEGF expression was assessed after incubating slides with mouse

anti human VEGF C1 monoclonal antibody (sc-7269, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). The antibody used reacts with VEGF of mouse, rat and human origin. Semi-quantitative scoring of both pimonidazole staining and VEGF expression was based on a method modified after Coppola et al.19 with a scale ranging from 0 to 6. The scale was based on scoring of the fraction of positive cells (0: all cells negative; 1: <33% positive; 2: 33-66% positive; 3: >66% positive) and the staining intensity (1: weak; 2: moderate; 3: intense). Both scores were added to a maximum score of six.

5.3.8 Statistical Analysis

Data were analyzed and presented graphically with a statistical software package (S-PLUS 6.1 for Windows, Insightful Corp., Seattle, USA). Differences in signal enhancement parameters between 4 tissue regions were evaluated with one way analysis of variance (ANOVA). Differences in imaging parameters and pO2 before versus after RT were assessed with the paired-sample t-test or, when data were not normally distributed, with the Wilcoxon signed rank test. Differences in MVD between RT treated rats and control rats were analyzed with the unpaired t-test, while differences in pimonidazole staining score and VEGF expression were analyzed with the Mann Whitney U test. Correlation analyses between imaging, oxygenation and histological data were performed with the Spearman rank order test. Statistical significance was inferred when $p < 0.05$. Data are expressed as mean with 95% confidence interval unless stated otherwise.

5.4 Results

5.4.1 Effect of RT on Tumor Growth

The mean cross-sectional surface area of the tumor did not change significantly during RT: 1.5 (0.9-2.1) cm2 before RT and 1.6 (0.8-2.4) cm2 after RT (p=0.9).

5.4.2 Enhancement of Tumor versus Normal Tissue

A similar temporal enhancement pattern was observed in all animals (Fig 5.2 and 5.3) confirming selective MRI enhancement of tumor tissue by P792. Before RT, the arterial input function tended to peak at approx. 100-120 seconds. Signal enhancement in neoplastic tissue was characterized by a short initial rapid increase of signal intensity (SI) corresponding to the first arterial inflow and initial rapid inwash in the tumor tissue. This 20-25 second phase was followed in tumor tissue by a slowly rising SI increase, which was most pronounced in the tumor rim. The tumor center displayed a similar slow uptake although the SI was much lower. Normal muscle, however, did not enhance after the initial fast uptake phase.

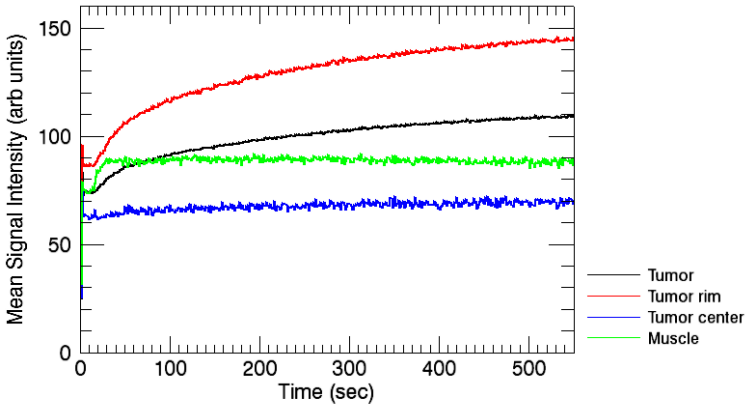

Figure 5.2: Typical enhancement pattern in different colorectal tumor regions and normal muscle before fractionated radiotherapy.

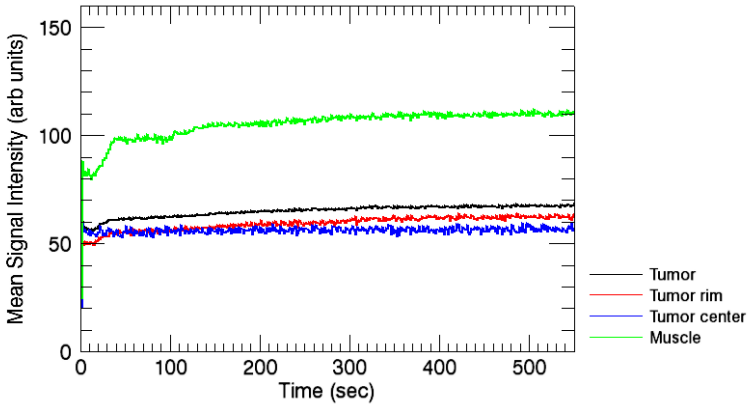

Figure 5.3: Typical enhancement pattern in different colorectal tumor regions and normal muscle after fractionated radiotherapy.

Cumulative DCE parameter values before RT are illustrated in Fig 5.4-5.6. Mean AUC of signal intensity was significantly different between entire tumor, tumor rim, tumor core, and normal muscle ($p<0.001$ for all comparisons). Both pharmacokinetic parameters were significantly different between the entire tumor, tumor rim and tumor core ($p<0.001$ for all comparisons), with the highest Ktrans observed at the tumor rim. No significant differences in pharmacokinetic parameters were seen between the tumor core and normal muscle ($p=0.4$ for both Ktrans and Ve).

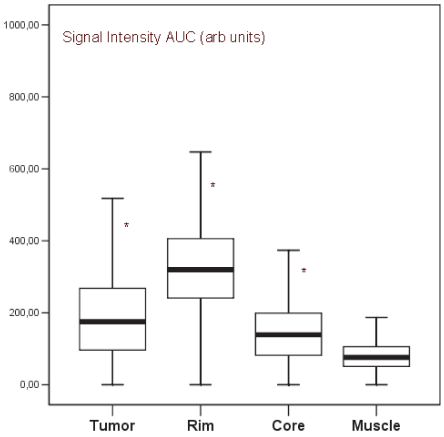

Figure 5.4: cumulative area under the concentration time curve values before radiotherapy in different regions of interest.

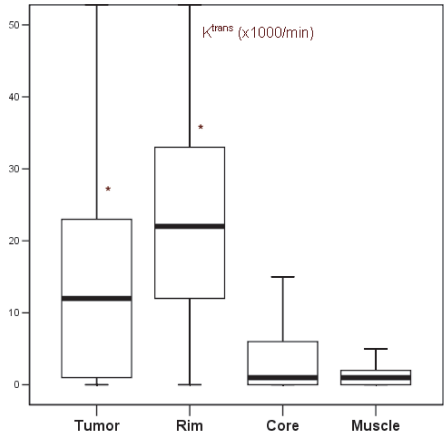

Figure 5.5: cumulative Ktrans (endothelial transfer constant) values before radiotherapy in different regions of interest.

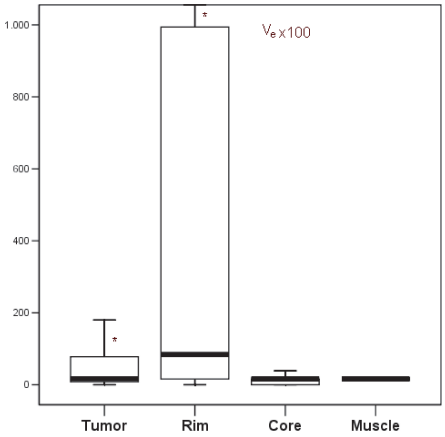

Figure 5.6: cumulative Ve (fractional interstitial space) values before radiotherapy in different regions of interest.

5.4.3 Effects of RT on DCE-MRI Parameters

The effects of fractionated RT were first evaluated graphically with parametric maps of the tumor (Fig 5.7). Both Ktrans and Ve were significantly lower after RT in all three examined regions of the tumor (Table 5.1). In non irradiated muscle tissue, on the contrary, no changes were observed in Ktrans or Ve.

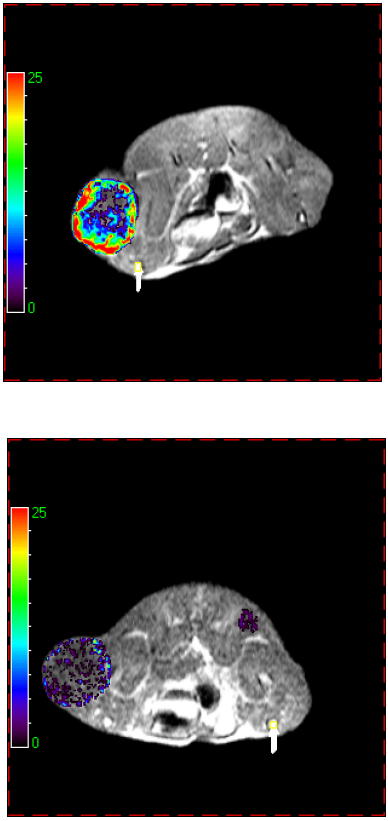

Figure 5.7: Parametric map (tumor masked) of Ktrans (x1000/min) before (above) and 5 days after (below) 5x5 Gy of radiotherapy. The arrow points to the pixel containing the selected arterial input function.

Parameter	ROI	before RT	After RT	p
Ktrans (min-1)	Tumor	14.6	3.8	<0.0001
	Rim	26.3	4.5	<0.0001
	Core	5.4	1.8	<0.0001
	Muscle	3.3	2.1	0.12
Ve	Tumor	20.3	2.4	<0.0001
	Rim	32.7	5.8	<0.0001
	Core	6.3	0.5	<0.0001
	Muscle	1.2	0.8	0.41

Table 5.1: Comparison of DCE-MRI parameters before and after radiotherapy in selected regions of interest (ROI).

5.4.4 Effects of Fractionated RT on Tissue pO2

The mean number of pO2 readings per animal was 839 before RT and 706 after RT. Mean pO2 values in both tumor regions before and after RT are illustrated in Table 5.2 and Fig 5.8 and 5.9. Both before and after RT, the tumor core was significantly more hypoxic compared to the tumor rim. Fractionated RT significantly increased mean pO2 in both tumor core and rim. The hypoxic fraction (pO2 <5 mm Hg) in the tumor core was 79.2% before and 61.9% after RT ($p = 0.008$). In the tumor rim, the hypoxic fraction was 36.6% before RT and 29.9% after RT ($p = 0.3$). The histogram of cumulative pO2 readings showed a bimodal distribution in both tumor regions.

	pre RT	post RT	
	mean (95%CI)	mean (95%CI)	p
Tumor rim pO2 (mm Hg)	6.8 (6.7-6.8)	7.7 (7.6-7.8)	<0.001
Tumor core pO2 (mm Hg)	3.5 (3.4-3.5)	4.4 (4.3-4.4)	<0.001
p	<0.001	<0.001	

Table 5.2: Oxygenation values in the tumor core and rim before and after radiotherapy. CI, confidence interval.

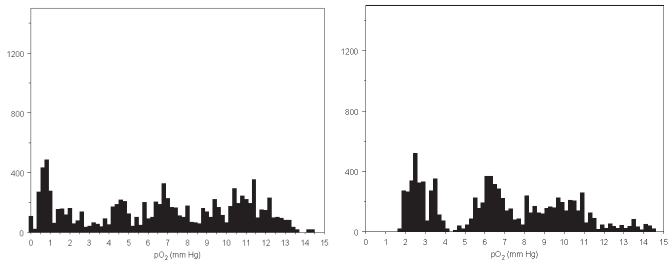

Figure 5.8: pO2 histograms from the tumor rim before (left) and after (right) radiotherapy.

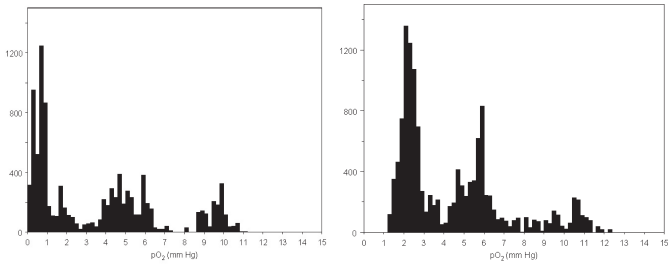

Figure 5.9: pO2 histograms from the tumor core before (left) and after (right) radiotherapy.

5.4.5 Immunohistochemistry Results

Expression of VEGF was significantly higher in RT treated rats in both tumor rim and core (Table 5.3). The pimonidazole hypoxia score, however, did not significantly differ between RT treated and control animals. In the tumor rim, mean MVD was lower in RT treated animals, but the difference did not reach statistical significance. In the tumor core, MVD was significantly lower compared to the tumor rim but did not differ between control and RT treated animals.

5.4.6 Correlation of DCE-MRI Parameters with pO2 and Histology

After RT, pO2 in the tumor rim was inversely related to Ktrans ($r = -0.57$, $p = 0.09$) and Ve ($r = -0.65$, $p = 0.04$) (Figure 5.10).

		Controls	RT	p
VEGF mean score	rim	2	5	0.005
	core	2	5	0.018
Hypoxia median score	rim	4	3.5	0.33
	core	4.5	4	0.79
MVD mean	rim	16.9	10.4	0.061
	core	5.3	6.1	0.33

Table 5.3: Immunohistochemistry scores in the tumor rim and core in control and irradiated animals. MVD, mean vessel density; RT, radiotherapy.

No correlation, however, was found between pO2 and signal intensity AUC after RT. Similarly, no significant correlations were found between DCE-MRI parameters and histological parameters (MVD, VEGF expression, pimonidazole hypoxia score).

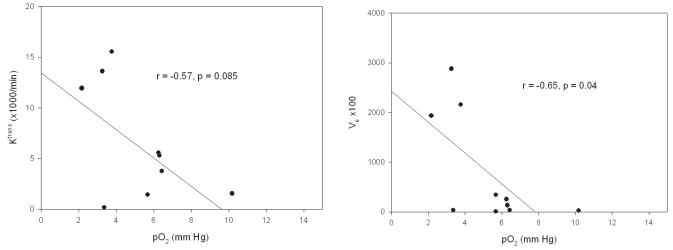

Figure 5.10: Correlation between pharmacokinetic parameters and pO2.

5.5 Discussion

Tumor vessels display ultrastructural abnormalities including transcellular openings, widened interendothelial junctions, abnormal endothelial cells, and absent basement membrane which increase permeability even for large molecules.[20]
In this study we examined the use of P792, a new macromolecular CA to measure changes in neovascular leakage induced by fractionated short term RT. Neovascular leakage is an important physiological tumor parameter that has been shown to respond quickly and dramatically to anti-angiogenesis interventions.[21] Leakage of contrast agent is determined both by endothelial permeability and by the total exchange surface area (proportional to the number of functional vessels). Microvessel counts were performed to differentiate permeability effects of RT from changes in the total surface area. In order to optimize imaging parameters describing the contrast agent dynamics, a high temporal resolution scanning method was used with contrast administered as a rapid central venous bolus.[22] DCE-MRI was compared with invasive pO2 mapping. The fluorescence lifetime method we have used to measure pO2 has several advantages over the Eppendorf device, including absence of oxygen consumption and increased accuracy at low oxygen tensions common in neoplastic tissue.[23] We chose to study post-RT effects relatively early to reflect clinical practice, with surgery after short term RT being usually performed within 1 week after the end of therapy.
Our main finding is a significant reduction of Ktrans after short term fractionated RT. Although the MVD did not differ sig-

nificantly between RT treated and control animals, a trend towards lower MVD in irradiated rats was present. Although the present analysis of a small number of animals does not allow us to draw a definitive conclusion, probably both altered endothelial permeability and a change in total microvascular surface area contribute to the decreased Ktrans after RT. Expression of VEGF was significantly higher in RT treated rats compared to control animals. Hypoxia as a trigger of VEGF expression was decreased after RT in this model. However, other radiation-induced activators of VEGF expression such as the mitogen activated protein kinase (MAPK) pathway have been shown to enhance VEGF expression following RT.[24] Overexpression of VEGF after RT has also been clinically demonstrated in rectal cancer patients.[25]

Published experimental data suggest that, depending on the dose and fractionation of RT and the timing of permeability measurement, neovascular permeability can be either increased or decreased.[26] Large, single RT doses disrupt the endothelial lining and cause a short term increase in endothelial permeability. Smaller or fractionated doses, however, tend to decrease vacular permeability or cause no change in this parameter. Several authors have used DCE-MRI to study the effects of RT on microvascular phyiology. Yu et al. used DCE-MRI with gadopentetate dimeglumine (Gd-DTPA) and the macromolecular CA gadomer-17 to study microvascular permeability in a rat adenocarcinoma model.[27] They observed a 67% decrease in permeability measured using gadomer-17 at 3 days after a single dose of 5 Gy, while no change was observed after a dose of 20 Gy. No changes in permeability were observed with either dose

of RT when Gd-DTPA was used as a contrast agent. Kobayashi et al. studied neovascular permeability with a macromolecular CA after a single dose of RT or fractionated RT.[28] They found an increased permeability after a single dose of 15 Gy, while permeability was unaffected by fractionated RT.
Several clinical studies have used DCE-MRI with small molecular weight MRI contrast agents to study tumor microenvironment during rectal cancer therapy. George et al. found a significant relation between pretreatment Ktrans assessed with Gd-DTPA and response to neoadjuvant chemoradiation.[29] Responsive tumors showed a marked reduction in Ktrans at the end of treatment (mean logarithmic Ktrans -0.46 versus 0.86; p=0.04). It should be noted that with the use of Gd-DTPA Ktrans probably represents both permeability and microvascular flow. Gd-DTPA as a contrast agent was also used by de Vries et al, who monitored microcirculation during chemoradiation for rectal cancer.[30] They calculated a perfusion index from the shape of the arterial and tumor tissue curves and found this value to be significantly increased up to two week after the start of chemoradiation. In patients treated with surgery only, Tuncbilek et al. found a significant relation between pretreatment descriptive DCE-MRI parameters and microvessel density, tumor grade and patient outcome.[31] We found that the biophysical and imaging properties of P792 allow to selectively study neoplastic vascular physiology and to monitor the effects of RT on tumor microvasculature. Several authors have used P792 to characterize tumor physiology in animal models. Turetschek et al. found no relation between transendothelial permeability estimated with P792 and histological parameters (microves-

sel density and tumor grade) in a breast cancer model.[32] In a prostate cancer model, however, P792 based DCE-MRI was successfully used to monitor changes in permeability following anti VEGF therapy.[15] It is likely, therefore, that succesful estimation of neovascular permeability with P792 is highly dependent not only on imaging and image processing methodology but also on tumor type and grade. A comparison of gadopentetate dimeglumine and P792 to characterize tumor physiology and metastatic ability in a rodent prostatic cancer was published by Fan et al.[33] They used an empirical mathematical model to fit the observed enhancement curves. In agreement with our findings, uptake of P792 was slow and influenced primarily by capillary leakage, with a strong uptake difference between tumor and normal tissue.

We combined DCE-MRI with invasive pO2 measurements, and found a significant increase in pO2 in the peripheral region of the tumor that was sampled with a fiberoptic probe. The available evidence concerning the effect of fractionated radiation on tumor oxygenation suggests that either an increase or a decrease in oxygenation can be observed depending on RT dose, fractionation, tumor histology, and timing of pO2 measurement.[34] Increased oxygenation shortly after fractionated radiotherapy has been previously reported and is the net result of changes in both oxygen supply (reduced interstitial pressure, increased flow) and oxygen consumption (decreased cell density).[35] Our results suggest a bimodal distribution of tumor pO2 values, a finding previously reported in a prostate cancer model using a noninvasive assay.[36] Oxygenation was sampled over a 4 mm trajectory; the observed bimodal distribution therefore probably

represents intralesion heterogeneity with severely hypoxic zones and better oxygenated zones surrounding feeding vessels. Before RT, no relation could be demonstrated between pO2 measurements and DCE-MRI parameters in the tumor rim. After RT, however, high pO2 values corresponded with low Ktrans and Ve values while no relation was found with the signal intensity AUC. This finding probably reflects the stabilizing effect of RT on tumor vasculature with a partial return of the physiological relationship between well oxygenated tissue and a structurally normal microvessel wall. Moreover, our data support the use of pharmacokinetic modeling of dynamic MRI data to generate parameters which directly reflect physiological processes in contrast with mere description of the tissue enhancement curve. In conclusion, the biophysical properties of P792 allow noninvasive measurement of microvascular leakage in a colorectal tumor model. Fractionated RT markedly decreases contrast leakage by altered neovascular permeability. After RT, reoxygenation of tumor tissue corresponds with a lowered transfer constant and leakage space. DCE-MRI could therefore be a tool in noninvasive monitoring of tumor microvascular response to fractionated RT. Future work in this model will combine RT with chemotherapy and targeted therapy.

5.6 References

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Chapter 6

Recombinant Human Erythropoietin α modulates the Effects of Radiotherapy on Colorectal Cancer microvessels

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6.1 Abstract

Purpose: to study modulation of radiotherapy (RT) effects on CC531 colorectal cancer microvessels by administration of recombinant human erythropoietin (rhEPO) in a WAG/Rij rat model.
Experimental Design: tumor bearing rats were administered 3x0.1 ml of rhEPO weekly. Five days before and after 5x5 Gy of RT, dynamic contrast enhanced (DCE) MRI was performed with P792. Mathematical modelling resulted in parametric maps of endothelial permeability surface product (PS), plasma flow (F), and blood volume (V). Imaging was combined with pO2 and laser doppler flow (LDF) measurements. After the second set of measurements, tumors were analysed for microvessel density (MVD), diameter and fractal dimension (MFD). Expression of VEGF, HIF-1α, Bax, and Bcl-2 was determined immunohistochemically.
Results: RT significantly reduced PS and V in control rats, but not in rhEPO treated rats, while F was unaffected by RT in both groups. Oxygenation was significantly better in rhEPO treated animals, and RT induced a heterogeneous reoxygenation in both groups. LDF was significantly lower following RT in the central tumor region of control rats, while no changes in LDF were seen in rhEPO treated rats. Microvessel diameter was significantly larger in rhEPO animals, while MFD was lower in the tumor core. VEGF expression was significantly lower in the rhEPO group. No differences were observed in HIF-α, Bax, or Bcl-2 expression.
Conclusions: rhEPO results in spatially heterogeneous mod-

ulation of RT effects on tumor microvessels. Direct effects of rhEPO on neoplastic endothelium are likely to explain these findings in addition to indirect effects induced by increased oxygenation.

6.2 Introduction

Anemia commonly occurs in colorectal cancer patients especially if they are treated with neoadjuvant radiotherapy (RT) or chemotherapy. Anemia not only adversely affects the clinical condition of these patients but also contributes to the development of tumor hypoxia, recognized as a major negative determinant of sensitivity to RT, chemoradiotherapy, and certain chemotherapeutic agents.[1,2]
Recent clinical studies have shown that administration of recombinant human erythropoietin (rhEPO, epoetin α) increases hemoglobin levels and improves quality of life in patients with cancer related anemia.[3] Over the last decade it has become clear that the action or rhEPO extends into a wide range of cellular mechanisms involved in stem cell development, maintenance of cellular integrity, and physiological angiogenesis.[4] The demonstration of the EPO receptor in various neoplastic tissues and the observation in a recent clinical trial that mortality was higher in non-anemic rhEPO treated breast cancer patients highlighted the possible effects of rhEPO on tumor growth and angiogenesis.[5] Preclinical studies investigating the role of EPO and EPO - EPO receptor signalling on tumor growth and angiogenesis have yielded contradictory results. Yasuda et al. noted

inhibition of angiogenesis and tumor cell survival in stomach and melanoma xenografts following blockade of EPO signalling.[6] The results of Hardee et al., however, suggested that administration of rhEPO did not affect angiogenesis or tumor growth in human colon and head and neck xenografts.[7]

The importance of tumor oxygenation for RT response is well established, and there has been considerable interest in modulating tumor oxygenation and RT response by rhEPO administration. Experimentally, exogenous rhEPO has been shown to improve or restore radioresponsiveness in both anemic and non-anemic animals.[8-10] Interestingly, darbepoetin α, an EPO analogue with a longer half-life, did not enhance radioresponsiveness in a rat mammary adenocarcinoma model.[11]

The exact mechanism by which rhEPO exerts its effects on tumor oxygenation is at present unclear. Indeed, recent data suggest that this effect may be independent of changes in hemoglobin and mediated by changes in vascular endothelial growth factor (VEGF) expression and microvessel morphology.[12,13]

We aimed to further characterize the effects of rhEPO on microvascular morphology and function in non-anemic rats using a novel imaging methodology. We previously used dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) with a macromolecular contrast agent to demonstrate a significantly decreased neovascular leakage after fractionated RT in a rat colorectal cancer model.[14] DCE-MRI allows non-invasive in vivo study of microvascular properties of a complete tumor, thereby taking into account the important spatial heterogeneity of solid tumors with zones of well perfused tissue as well as hypoxic or necrotic areas.[15] We here studied the effects of rhEPO on RT-

induced microenvironmental changes in a rat colorectal cancer model and correlated non-invasively obtained data with invasive oxygenation and flow measurements, microvessel density, complexity and diameter, and expression of hypoxia-regulated and apoptosis markers.

6.3 Materials and Methods

The experimental protocol was approved by the Animal Experimentation Ethical Committee of the Ghent University, Ghent, Belgium.

6.3.1 Animal and tumor model

Male Wag/Rij rats were bought from Harlan, Horst, The Netherlands. The CC531 cell line is a 1,2-dimethylhydrazine-induced, moderately differentiated and weakly immunogenic colon adenocarcinoma, syngeneic with WAG/Rij rats. This cell line is well studied and has been proven to provide a tumor-host model similar to human colorectal carcinogenesis.[16] Cells were grown in plastic culture flasks in RPMI 1640 medium, buffered with HEPES (20 mM) (Invitrogen Corporation, Gibco, Ghent, Belgium) additionally supplemented with 10% fetal calf serum, 4mM L-glutamine, 50 U/ml penicillin and 50 g/ml streptomycin at 37 degrees C in a humidified atmosphere with 5% CO2 in air. The cells were transferred at 95% confluency. Two million cells suspended in 0.2 ml of saline were injected subcutaneously in the proximal hind leg. Tumors reached a size of 0.5-1 cm af-

ter a period of 4 weeks. Once a tumor growth of minimally 8 mm diameter was observed, a jugular vein catheter was inserted and tunneled to the interscapular region. In order to maintain catheter patency, continous infusion at 0.5 ml saline per hour was administered with a cage mounted swivel and flexible metal tether system (Uno BV, Didam, The Netherlands) allowing the animal full mobility.

6.3.2 Experimental therapy

Recombinant human EPO has been shown to bind to the rodent EPO receptor.[17] Animals were randomly divided in 2 groups: a control group (n=11) and an rhEPO group (n=15) receiving rhEPO (Eprex, Janssen Cilag, Beerse, Belgium) at a dose of 3x0.1ml (286 IU) sc per week. The dosage was based on a dose finding study during which five or eight rhEPO administrations weekly resulted in an excessive hematocrit rise and important mortality (data not shown).
Rats were longitudinally studied during three weeks using the following timeframe: start of rhEPO administration (day 1); first DCE-MRI, oxygenation and flow measurement (day 8); fractionated RT 5x5 Gy (day 13-17); second DCE-MRI, oxygenation and flow measurement, and sacrification by anesthesia overdose and excision of tumors for histology (day 22).

6.3.3 Radiotherapy

Rats were not sedated and the tumor bearing hind leg was immobilized using a plexiglass holder, as described previously.[18,19]

Briefly, rats were placed in a purpose-built plexiglass holder in prone position. The hind legs were pulled through an opening in the holder and immobilized. Before each fraction, a radiation field was simulated encompassing the tumor with a margin of 1.5 cm. The photon irradiation was performed with a 5 MV linear accelerator (Elekta, Crawley, UK). Five fractions of 5 Gy (total dose 25 Gy) were delivered on five consecutive days. Since the tumors were inoculated subcutaneously, they were covered with tissue-equivalent silicone bolus of 1 cm to prevent the build-up effect under the skin. One single direct field at a fixed source-skin distance of 100 cm was used. The dose was calculated to the midpoint of the tumors according to their volume in each individual animal, as obtained during simulation. DCE-MRI and oxygenation measurements were performed 5 days before and 5 days after the completion of RT.

6.3.4 Magnetic Resonance Imaging

The principle of DCE-MRI consists of serial measurements of signal intensity changes in both tumor tissue and a feeding artery after bolus injection of a paramagnetic contrast agent (CA). Depending on the physical properties of the CA and the leakiness of the microvessel wall, a fraction of the CA will reach the interstitial space of the tumor where an increase in signal intensity over time will be observed. After translation of signal intensity changes to CA concentration values, pharmacokinetic modelling allows calculation of physiological properties such as microvessel permeability and tumor blood volume.
Dynamic contrast studies were performed with P792, a new

monogadolinated rapid clearance MRI blood-pool CA which is cleared by renal elimination. The molecular weight of the compound is 6.47 kDa, but the mean diameter of P792 is 50.5 Angstrom and the T1 relaxivity of this agent is 29 mM-1 s-1 at 60 MHz.[20] The apparent hydrodynamic volume of P792 is 125 times greater than that of Gd-DOTA (gadoterate meglumine, Dotarem) and as a result of this high molecular volume, P792 is characterized by a limited diffusion across normal endothelium and therefore ideally suited to study hyperpermeable neoplastic vessels.[21]

Experimentally, P792 has been used to study permeability effects of anti-angiogenesis therapy in a prostate cancer model.[22] We have previously demonstrated that P792 selectively enhances tumor tissue in this colorectal cancer model.[14] T1 weighted DCE-MRI was performed on a Siemens Magnetom Symphony 1.5 Tesla scanner (Siemens AG, Erlangen, Germany). Animals were sedated with 0.2-0.4 ml of medetomidine (Domitor, Novartis Animal Health, Basel, Switzerland). Imaging comprised a single axial slice that was positioned through both lower limbs and the center of the tumor. Prior to the contrast series, T1 zero time maps were constructed from two spin echo sequences with different repetition times (TR 1000 ms and 318 ms, respectively). Details of this sequence were as follows: slice thickness 3 mm, field of view (FOV) 140x88, matrix size 256x160, echo time (TE) 20 ms, and flip angle 90 degrees. Dynamic imaging was performed with a 4 antenna wrist coil (diameter 10 cm) using an inversion recovery TurboFLASH sequence. Details of the pulse sequence were as follows: temporal resolution 1.1s, FOV 140x88, matrix size 256x160, slice thickness 5 mm, TE 4.08 ms,

inversion time 560 ms, and flip angle: 12 degrees. A bolus of 0.3-0.4 ml of P792 was manually injected as fast as possible (approximately 1ml/s) through a central venous line after the fourth scan. A total of 500 images was obtained for a total scan time of 550 seconds.

6.3.5 Tracer Kinetic Modelling

Pixel by pixel pharmacokinetic modelling of DCE-MRI data was performed with the research mode of a dedicated software package (MIStar, Apollo Medical Imaging, Melbourne, Australia). Extraction of both microvascular permeability and flow data was based on the tissue homogeneity (TH) model of capillary exchange originally described by Johnson and Wilson and later adapted for the study of cerebral flow by St Lawrence and Lee.[23,24] This model (Fig 6.1) consists of a plasma space, in which the contrast agent concentration is a function of both time and distance along the capillary unit, and an extracellular extravascular space (EES) assumed to be homogenously mixed (ie, a compartment). Leakage of contrast agent takes place between the vascular space and EES through a semipermeable membrane characterized by a permeability surface product (PS). Since P792 does not enter the intracellular compartment, the sum of the fractional plasma volume (Vp) and fractional extracellular volume (Ve) reached by the CA equals 100%, ie Vp+Ve=1.
When the assumption is made that changes in EES concentration per unit time are negligible compared to changes in plasma concentration, an adiabatic approximation to the TH model can

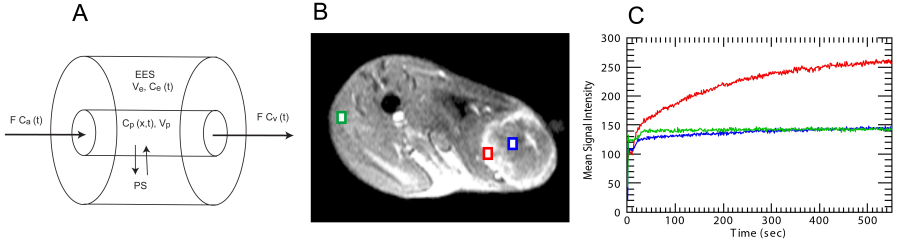

Figure 6.1: Illustration of the St Lawrence and Lee tissue homogeneity model applied to dynamic MRI data. A, two compartment model used for kinetic modelling. Following injection, contrast agent (CA) enters the tissue capillary unit (length x) and distributes between the vascular space and the extracellular extravascular space (EES) through a semi-permeable vessel wall characterized by a permeability surface product PS. Measurement of the temporal changes in CA concentration (calculated from changes in MRI signal intensity) in both the vascular space and the EES allows to calculate the plasma flow (F), fractional vascular volume Vp, and PS. B and C, Examples of contrast enhancement curves (500 images with 1.1 sec temporal resolution) observed in regions of interest selected in the tumor rim (red), tumor core (blue), and normal muscle. Normal microvessels do not leak the macromolecular contrast agent. The colorectal cancer is grown subcutaneously in the left lower limb.

be derived which has been used in modelling of DCE-MRI contrast agent kinetics applied to tumor microvasculature.[24,25] The adiabatic approximation to tissue homogeneity (AATH) model is given by superposition of the arterial input function (AIF) with a time varying residue function:

$$C_e(t) = F_p C_a(t) \otimes R(t)$$

where Ce (mM) is the contrast agent (CA) concentration in the EES, Fp (unitless) is the plasma flow, R denotes the residue function, and $\otimes$ the convolution operator. The time course of CA arrival is divided in a vascular phase (t$<\tau$, with τ the transit time through a capillary) and a tissue phase (t$>\tau$). Depending on the time interval, the residue function will encompass a vascular and a tissue component:

$$\begin{aligned} R(t) &= 1 & 0 \leq t < \tau \\ R(t) &= Ee^{\frac{-K^{trans}}{v_e}(t-\tau)} & t \geq \tau \end{aligned}$$

with E the extraction ratio from plasma space to EES, Ktrans the endothelial transfer constant (min-1),Ve the fraction of the EES available as leakage space (unitless), and τ the mean capillary transit time (s). The tissue contrast agent concentration can therefore be modelled as:

$$C_e = F_p \int_0^\tau C_a(t-t')dt' + K^{trans} \int_\tau^t C_a(t') e^{\frac{-K^{trans}}{v_e}(t-t'-\tau)} dt'$$

In each animal, the pixel containing the AIF curve was selected in the femoral artery feeding the tumor bearing limb. A region of interest (ROI) was drawn encompassing the outer vascular rim of each tumor. Within this ROI, a pixel by pixel curve fitting routine based on the Levenberg-Marquardt minimization method was performed generating parametric maps of the following parameters: Fp, PS, and the fractional plasma volume Vp, calculated as Fp x MTT. Numerical parameter values for each pixel were exported to a spreadsheet for statistical analysis.

6.3.6 Tissue pO2 and flow measurements

Tissue oxygenation and laser doppler flow (LDF) were measured with a fiberoptic probe combining fluorescence quenching with laser doppler flowmetry (OxyLite and OxyFlo, Oxford Optronix, Oxford, UK).[12,26] A precalibrated fiberoptic probe was inserted 5 mm deep into the tumor using a Seldinger technique; the probe was then withdrawn in 40 steps of 100 μm each over a total distance of 4 mm using a micromanipulator (model MN151, Narishige International Ltd, London, UK). After each micromanipulator movement, measurements were started as soon as a stable reading was obtained. Tissue pO2 was sampled every 2 seconds. Over this 4 mm trajectory, oxygenation and LDF values were recorded separately for the tumor core (central 1-2 mm) and peripheral angiogenic rim (outer 1 mm). Tissue pO2 was expressed in mm Hg while LDF was expressed in arbitrary units.

6.3.7 Immunohistochemistry

Paraffin-embedded tissue samples were used for immunohistochemistry with the following antibodies: anti-EPO receptor (M-20) (sc-697, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), anti-Bcl-2 (sc-7832, Santa Cruz), anti-Bax (sc-7480, Santa Cruz), anti-VEGF (sc-7269, Santa Cruz) and anti-HIF-1alfa (sc-10790, Santa Cruz). Paraffin-embedded sections were rehydrated by serial immersion in xylene and ethanol. After rinsing, the endogenous peroxidase was blocked with 0.3% hydrogen peroxide. The sections were subsequently incubated with a biotinylated secondary antibody, followed by incubation with a streptavidin-peroxidase complex (LSAB+ kit, Dako). The color reaction was developed using 3-amino-9-ethylcarbazole substrate (Dako) as chromogen. Finally, the sections were counterstained with hematoxylin. Semi-quantitative scoring was based on a method modified after Coppola et al. with a scale ranging from 0 to 9.[27] The scale was based on scoring of the fraction of positive cells (0: all cells negative; 1: <33% positive; 2: 33-66% positive; 3: >66% positive) and the staining intensity (1: weak; 2: moderate; 3: intense). Both scores were multiplied to a maximum score of 9. Scoring was performed separately on the tumor core and peripheral tumor rim.

6.3.8 Microvascular Density and Diameter

Microvascular density (MVD) was determined with a method modified after Weidner et al.[28] After incubating 5 μm frozen slices with anti-CD31 antibodies (TLD-3A12, Serotec, Oxford,

UK), the entire tumor section was scanned at low power (objective, 4 X) to identify 'hot spots', which are the areas of highest neovascularization. Individual microvessels were then counted under higher power (objective, 40 X) to obtain a vessel count in a defined area, and the average vessel count in 3 hot spots was taken as the MVD.
Microvascular diameter was measured on digitized CD31 stained slices (objective, 10 X). From each rat, five different zones were analysed and the largest diameter measured from all visible microvessels using NIH ImageJ software (version 1.35p, available from http://rsb.info.nih.gov/ij).

6.3.9 Microvessel Fractal Dimension

The tumor associated microvascular network can be considered as a complex architecture defined not only by the number of microvessels but also by the degree of branching, tortuosity and irregularity. Fractal analysis of two dimensional histology slides has been shown to provide additional information on tumor microvascular complexity.[29,30] Whereas classical geometrical objects are usually associated with integer values for a dimension (1 for a line, 2 for a square), complex biological structures are best defined by a fractal dimension that is a rational number between 1 and 2. The more complex (branched, tortuous) the microvascular structure, the closer the fractal dimension is to 2. From each tumor, digital images were obtained from five CD31 stained tumor hot spots (objective, 10 X) and analysed with ImageJ software. By applying a color threshold, non CD31 stained pixels were removed from the image. The microvessel

fractal dimension (MFD) was calculated using the box counting method. The image is divided into increasingly smaller boxes, and after each step the number of non empty boxes is counted. The fractal dimension is calculated as

$$D = \lim_{\varepsilon \to 0} \frac{\log N(\varepsilon)}{\log(1/\varepsilon)}$$

with ϵ the length of a box side, and N (ϵ) the smallest number of boxes required to contain all CD31 stained pixels. In practice, the limit of a box sized 0 cannot be applied and therefore the MFD was calculated as the slope of the curve fitted after plotting log N (ϵ) versus log ($1/\epsilon$). Calculations were performed with the fractal dimension plugin available in the ImageJ environment.

6.3.10 Statistical Analysis

Data are expressed as mean standard error of the mean, unless stated otherwise. Differences between 2 groups of continuous data were analysed with the Student t-test or, when data distribution was non Gaussian, with the non-parametric Mann Whitney U test while differences between fractions were evaluated with the Chi square or Fisher exact test. Statistical significance was assumed when $p \leq 0.05$. All calculations and plotting were performed with SigmaStat software (version 3.11, Systat Software, Richmond, USA).

6.4 Results

6.4.1 Expression of the EPO Receptor

All tumors showed expression of the EPO-R on neoplastic cells and neoplastic endothelium (Fig 6.2). There was no difference in expression score between control and rhEPO treated animals. In both groups, however, EPO-R immunoreactivity was significantly higher in the tumor core compared to the vascular tumor rim.

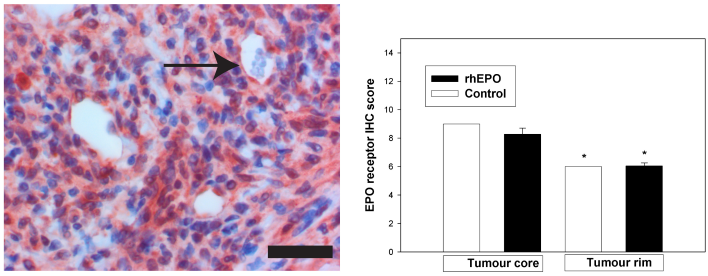

Figure 6.2: Immunoreactivity of the erythropoietin receptor. A, representative immunohistochemistry showing both cytoplasmatic and membrane staining of tumor cells and endothelial cells (arrow). Bar, 25 μm. B, comparison of EPO-R immunoreactivity scores in the tumor rim and core of both control and rhEPO treated animals. Columns, mean; Bars, standard error; *p < 0.001 versus the tumor core.

6.4.2 Effects of rhEPO on Hematocrit

Hematocrit values were tested before starting the experiment and on day 8 (immediately before the first MR imaging and oxygenation measurements). In rhEPO treated rats, mean hematocrit showed a 25% increase from 50.7% ± 0.4% before therapy to 62.6% ± 0.4% on day 8 ($p < 0.001$, Student t test). In the control group, hematocrit values remained unchanged.

6.4.3 Effects of rhEPO on Tumor Growth

The present experiment was intended to detect early microvascular changes after fractionated RT and not to study the effects of rhEPO on tumor growth or modulation of RT effects on tumor growth. Administration of rhEPO was therefore started after a tumor had developed. Tumor volume before RT was 1 cm^3 ± 0.2 cm^3 in the control group and 0.97 cm^3 ± 0.11 cm^3 in the rhEPO group ($p = 0.79$, Mann Whitney U test). After RT, tumor volume was 0.99 cm^3 ± 0.2 cm^3 in the control group and 0.99 cm^3 ± 0.16 cm^3 in the rhEPO group ($p = 0.99$, Mann Whitney U test).

6.4.4 Effects of rhEPO on RT Induced Microvascular Changes

Pixel by pixel kinetic modelling encompassing both the tumor core and rim was performed with estimation of 3 microvascular parameters: plasma flow Fp, permeability surface product PS, and fractional plasma volume Vp.

Results of DCE-MRI microvascular data modelling are shown in Fig 6.3. Microvascular plasma flow in the tumor core of control rats was significantly lower after RT ($p < 0.001$, Student t test). No significant effect of RT was noted in the tumor rim of control animals. In rhEPO treated rats, microvascular flow in the tumor core was also significantly lower after RT ($p < 0.001$, Student t test) but unaffected in the tumor rim. The magnitude of the RT effect on tumor core flow did not differ significantly between control and rhEPO treated animals (33% versus 23% decrease respectively, $p = 0.11$, Fisher exact test). In control animals, the microvessel permeability surface product (PS) was significantly lowered by RT in the tumor rim ($p < 0.001$, Student t test) but not in the tumor core. In rhEPO treated animals, however, PS was not affected by RT.
Fractional plasma volume was significantly lower in both the tumor core and rim in control animals after RT ($p < 0.001$, Student t test). In rhEPO treated animals, however, no significant changes in plasma volume were noted after RT.

6.4.5 Effects of rhEPO on Oxygenation and Flow

Oxygenation and laser Doppler flow measurements were performed before and 5 days after completion of fractionated RT. Mean pO2 values in the tumor core and peripheral rim before and after RT in both groups are shown in Fig 6.4 A and B. Oxygenation was significantly better in the tumor rim compared to the tumor core of all animals (data not shown). In the control group, RT induced a reoxygenation in the tumor core (p

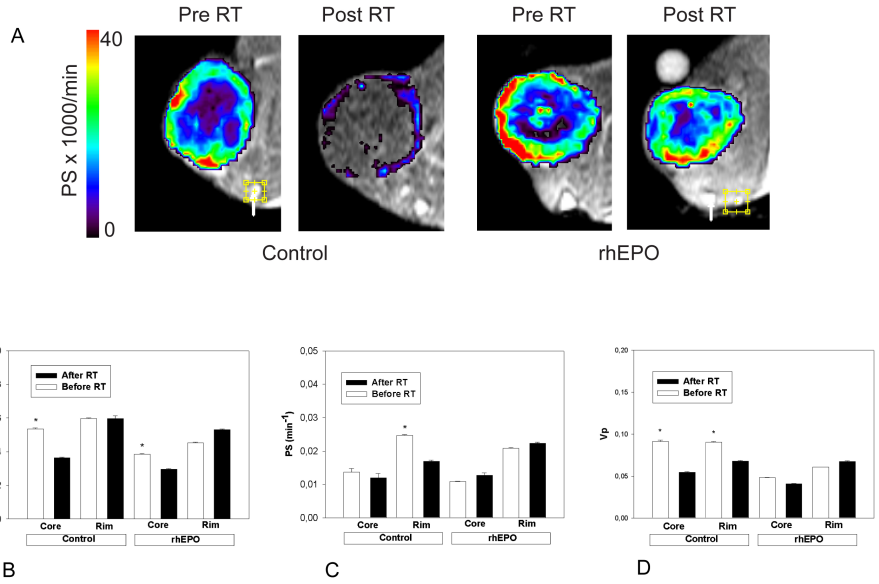

Figure 6.3: Results of kinetic modelling of DCE-MRI data. A, example of parametric maps of the permeability surface product (PS) in control animals and rhEPO treated animals before and after radiotherapy (RT). The tumor is masked and the arrow points to the pixel containing the selected vascular input function. B-D, combined kinetic data for microvascular flow (Fp), permeability surface product (PS), and fractional plasma volume (Vp). Columns, mean; Bars, standard error, *p < 0.001.

= 0.067, Student t test) but not in the tumor rim (p = 0.36, Student t test). In the rhEPO group, on the contrary, no significant difference in oxygenation was observed in the tumor core (p = 0.12, Student t test) while a significant increase in pO2 was observed in the tumor rim (p = 0.032, Student t test). Both before and after RT, pO2 values were significantly higher in rhEPO treated rats in both regions of the tumor (data not shown). Mean LDF values (arbitrary units) are illustrated in Fig 6.4 C. In all animals, LDF was significantly higher in the tumor rim compared to the tumor core. In the control group, RT significantly decreased LBF in the tumor core (p = 0.023, Student t test) but not in the tumor rim. In the rhEPO group, however, RT did not influence LDF in either tumor zone. Before RT, LDF measured in the tumor core was significantly lower in rhEPO treated animals (p = 0.014, Student t test) compared to controls while no significant difference was present in the tumor rim. After RT, LDF values in both tumor core and rim were not significantly different between control and rhEPO treated animals (data not shown).

6.4.6 Effects of rhEPO on Microvessel Density

Mean microvascular density (MVD) was 12.5 ± 1.2 in the control group and 14.3 ± 1.4 in the rhEPO group (p = 0.35, Student t test). Within each group, MVD was significantly lower in the tumor core compared to the tumor rim (12.5 ± 1.2 versus 7.3 ± 0.6, $p < 0.001$ in the control group and 14.3 ± 1.4 versus 6.6 ± 0.5, $p < 0.001$ in the rhEPO group, Student t test).

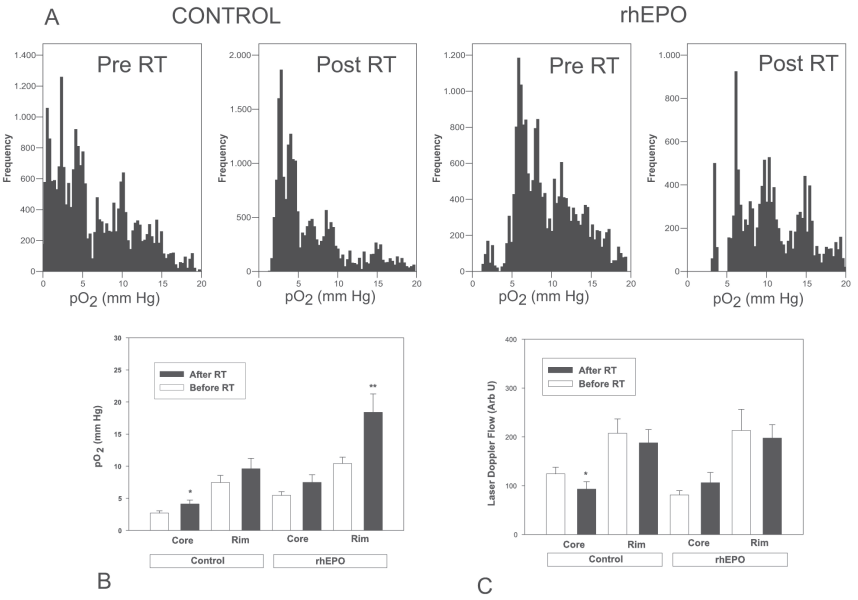

Figure 6.4: Combined results of invasive pO2 and laser Doppler flow measurements. Measurements were performed before and 5 days after the end of 5x5 Gray of RT. A, histograms of pO2 readings over a 4 mm trajectory in control and rhEPO treated rats both before and after RT. A reoxygenation occurs after the end of RT which is more pronounced in the rhEPO group. B, oxygenation data in the tumor core (central 1-2 mm) and tumor rim (superficial 1-2 mm). Columns, mean; Bars, standard error, *p = 0.067; **p = 0.032. C, laser Doppler flow data in the tumor core and rim. Columns, mean; Bars, standard error, *p = 0.023.

6.4.7 Effects of rhEPO on Microvessel Fractal Dimension and Diameter

Microvessel morphology data are illustrated in Fig 6.5. Microvessel fractal dimension was spatially heterogeneous. In the tumor core, MFD was significantly lower in rhEPO treated animals ($p = 0.006$, Student t test). In the tumor rim, however, MFD did not differ between control and rhEPO animals ($p = 0.62$, Student t test), Fig 6.5 C. Overall microvessel diameter (μm) in tumor tissue was 55.9 ± 2.1 in the control group and 75.4 ± 2.7 in the rhEPO group, $p < 0.001$. The increased microvessel diameter in rhEPO treated animals was more pronounced in the tumor core ($p < 0.001$, Student t test) than in the tumor rim ($p = 0.07$, Student t test), Fig 6.5 D.

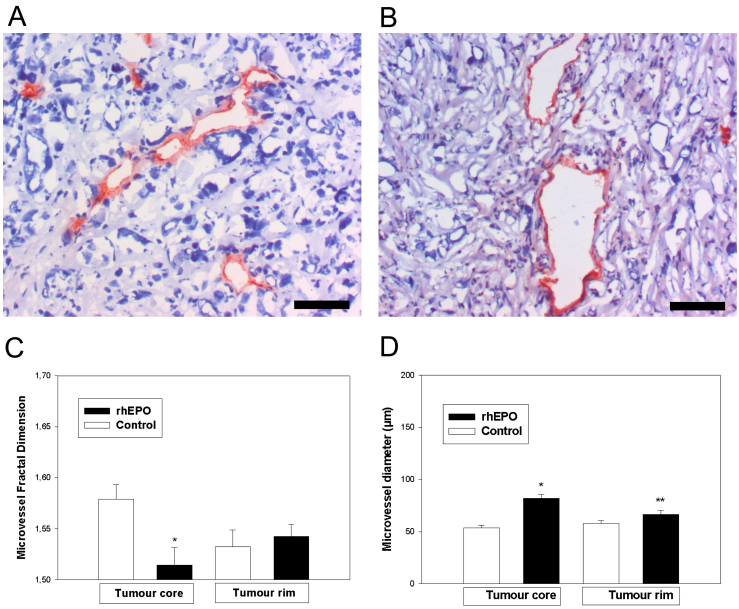

Figure 6.5: Comparison of tumor microvessel fractal dimension and diameter in control and rhEPO treated rats. A and B, examples of CD31 stained microvessels in the tumor core of control and rhEPO treated animals. Bar, 25 μm. C, microvessel fractal dimension in the tumor core and rim in control and rhEPO treated animals. Columns, mean; Bars, standard error; *p = 0.006. D, microvessel diameter in the tumor core and rim in control and rhEPO treated animals. Columns, mean; Bars, standard error; *p < 0.001; **p = 0.07.

6.4.8 Effects of rhEPO on Expression of VEGF, HIF-1alfa, Bax, and Bcl-2

Immunohistochemistry data are summarized in Fig 6.6. Total VEGF expression score was significantly higher in the control group ($p = 0.048$, Mann Whitney U test). Within each group, the difference in VEGF expression in the tumor core versus tumor rim was not significant. Total expression of HIF1α did not differ significantly between both groups ($p = 0.78$, Mann Whitney U test). There was also no significant difference in expression of Bax ($p = 0.21$, Mann Whitney U test test) or Bcl-2 ($p = 0.72$, Mann Whitney U test) between control and rhEPO treated animals. In rhEPO treated animals, Bcl-2 expression was significantly lower in the tumor core compared to the tumor rim ($p = 0.012$, Mann Whitney U test).

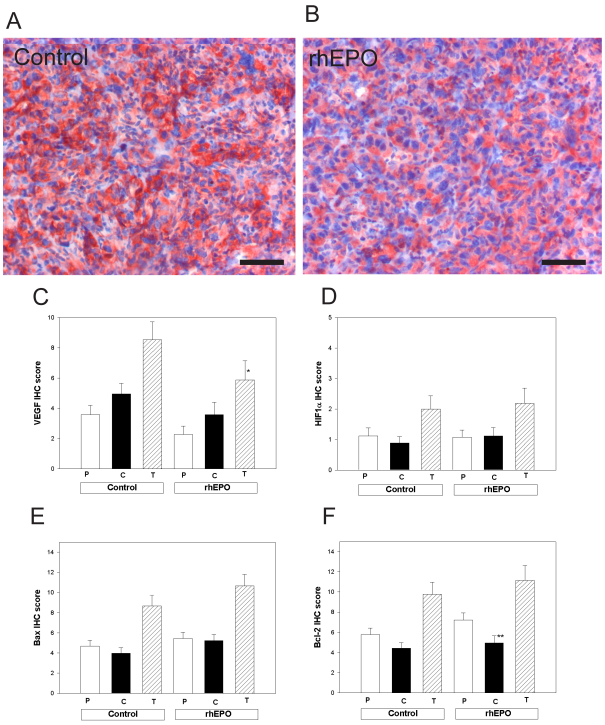

Figure 6.6: Expression of hypoxia regulated and apoptosis related markers. A and B, example of VEGF staining intensity in control and rhEPO treated animals. Bar, 50 μm. C-F, expression of VEGF, HIF-1α, Bax, and Bcl-2 in the tumor peripheral rim (P), core (C), and total score. Columns, mean; Bars, standard error; *p =0.048; **p = 0.012.

6.5 Discussion

The presence of hypoxia adversely affects radiotherapy response and prognosis in cancer patients.[31] Since the oxygen carrying capacity is mainly determined by the blood hemoglobin concentration, pharmacological manipulation aiming to restore or increase hemoglobin levels have received considerable interest in cancer patients undergoing RT or chemotherapy. Erythropoietin is a pleiotropic hormone whose biological role has recently been shown to extend not merely to the hematopoietic tissues but also to the neuronal and cardiovascular systems, where it exerts a cytoprotective effect.[4] Administration of exogenous rhEPO not only improved quality of life but increased survival in a number of clinical studies in solid tumors.[32,33] On the other hand, EPO has been shown to exert direct effects on angiogenesis and tumor growth mediated by presence of the EPO receptor on endothelial cells and a number of malignant cell types.[34,35] Preclinical studies investigating the effect of exogenous rhEPO on tumor growth and angiogenesis are at present inconclusive.[7] Similarly, while some preclinical data suggest that rhEPO increases response to RT, chemotherapy or photodynamic therapy, other studies did not identify any effects of rhEPO.[8,10,11] We aimed to study how rhEPO modulates the early in vivo effects of RT on colorectal cancer microvasculature. Undoubtedly, modulation of RT effects is mediated by both increased oxygenation and direct effects of rhEPO on normal and tumor microvessels. Moreover, solid tumors are characterized by an important heterogeneity with both well oxygenated and hypoxic or necrotic regions. Therefore, non-invasive functional

imaging was used that allows differentiating between different tumor regions. Expression of the EPO-R was present in all tumors and significantly more pronounced in the highly vascular tumor rim. There is at present no clearly defined relationship between tumor hypoxia and expression of the EPO receptor by cancer cells. In head and neck cancer patients, Arcasoy et al. found a positive correlation between tumor hypoxia and EPO-R expression while others did not observe any correlation in a similar patient cohort.[36-38] The experiment was not intended to study changes in macroscopic tumor growth. We analysed rhEPO mediated modulation of tumor cell sensitivity to apoptosis. In contrast to the findings of Batra et al., we did not observe any difference in expression of apoptotoc or anti - apoptotic markers between rhEPO treated animals and controls.[39] The spatial distribution of apoptotic events did, however, differ in rhEPO treated animals. In contrast to control animals, Bcl-2 expression in rhEPO treated animals was significantly different between tumor rim and core suggesting an increased efficacy of RT in the central region of the tumor by administration of rhEPO. Dynamic MRI with a macromolecular contrast agent is a validated technique to provide a comprehensive assessment of tumor microvascular physiology.[40,41] Pharmacokinetic two compartment modelling of DCE-MRI data was performed on regions of interest encompassing the tumor vascular rim and the tumor central core. Microvascular plasma flow was significantly decreased by RT in the tumor core (but not in the tumor rim) of both control and rhEPO treated animals. In keeping with previous preclinical and clinical findings [14], 5x5 Gy of RT decreased endothelial permeability (a surrogate marker for

angiogenesis) in the vascular tumor rim of control animals. In rhEPO treated animals, however, endothelial permeability was unaffected by RT. Similarly, while the tumor vascular volume was significantly lower after RT in both the tumor core and rim of control animals, no changes were present in rhEPO treated animals. This difference in response could be explained both by the previously described direct angiogenic potential of rhEPO counteracting the effect of RT and by the ability of rhEPO to remodel microvessels by an increase in diameter as confirmed by the microscopy data (cfr infra).[13,35] This modulation of RT effects was accompanied by a significantly lower expression of VEGF in rhEPO treated animals, a finding previously reported in colorectal xenografts.[13] Since no difference in HIF-1α expression was noted, the difference in VEGF expression is likely to result both from better oxygenation of the tumor rim and from direct effects of rhEPO on VEGF expression. Radiotherapy itself has been shown to induce changes in tumor tissue oxygenation. After a single dose of 20 Gy, Ljungkvist et al. observed a significant decrease in hypoxic fraction in a murine adenocarcinoma model.[42] We found the increase in oxygenation after 5x5 Gy to be spatially heterogenous. Interestingly, in rhEPO treated animals reoxygenation after RT mainly occurred in the tumor rim while in control animals reoxygenation of the core was more pronounced. Administration of rhEPO resulted in a significantly better oxygenation in both tumor regions compared to control animals. This effect is attributable to an increased blood oxygen carrying capacity and has been observed in other preclinical models.[10]
Erythrocyte flux was measured with the laser Doppler shift

method and, in contrast to the modelled plasma flow, provides additional information on rheological properties of the microvasculature. The observed lower LBF in the central region of rhEPO treated animals might be explained by a higher viscosity paralleling the difference in hematocrit.
In keeping with others [7], we were unable to demonstrate any effect of rhEPO on angiogenesis, as reflected by the similar MVD in both groups. Density of microvessels, however, is only one functional aspect of a tumor microvascular bed. Aspects such as morphology (tortuosity, branching pattern, microvessel diameter), maturation and endothelial wall permeability represent equally important attributes. We found a significantly larger microvessel diameter in rhEPO treated rats. Moreover, the microvessel fractal dimension was significantly lower in the central region of rhEPO treated rats, suggesting a lower spatial microvessel complexity.[43] These findings confirm the observation of Tovari et al. that rhEPO can 'remodel' tumor microvessels although their density seems unaffected.[13] The exact mechanism likely involves direct action of rhEPO on the endothelium rather than indirect effects mediated through changes in oxygenation, since VEGF expression was significantly lower in rhEPO treated compared to control animals.
In conclusion, the effects of RT on colorectal tumor microvascular physiology are spatially heterogeneous and modulated by administration of rhEPO. Treatment with rhEPO prevented RT induced changes in microvascular permeability and tumor vascular volume, accompanied by a larger microvessel diameter and altered spatial complexity compared to control animals. It is at present unclear whether this microvascular modulation increases

or counteracts the antitumoral efficacy of RT in this model. However, since rhEPO resulted in an increased oxygenation of the tumor rim, radiation response of the clonogens is likely to be increased by rhEPO, as described by others.[10]
Further preclinical experiments will have to elucidate the multiple molecular effects of rhEPO and the interaction with RT on tumor cells, neoplastic endothelium, and normal endothelium.

6.6 Acknowledgments

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Chapter 7

General Discussion

Colorectal cancer is a frequent malignancy in both men and women, and the ageing of the population will likely give rise to an important incidence increase in the decades to come. Despite efforts to introduce screening programs, most patients are diagnosed in a locally advanced stage of the disease. Locally advanced rectal cancer continues to present a technical challenge for surgical oncologists. In order to improve both local results and long term outcome, most patients are now treated in a multimodality fashion involving surgeon, radiotherapist, and oncologist. The aim of this work was to contribute to clinical and experimental aspects of multimodal therapy in rectal cancer. In **chapter three**, a systematic review of the literature on adjuvant and neoadjuvant therapy regimens is provided. From both expert series and national training programs, it is clear that improvements in surgical technique have resulted in important

improvements not only in LR rate but also in functional outcome and long term survival. In Belgium, national registry data indicated that, compared to published outcome data, important improvements can be achieved in terms of permanent colostomy rate, local recurrence rate, and long term survival.[1] This finding was the impetus for the ProCare (Project for Cancer of the Rectum) initiative, a nation wide training program of quality control and improvement involving surgeons, pathologists, and radiation oncologists. Meta-analyses have shown that preoperative RT alone significantly improves LR rate and marginally improves survival, provided the biologically equivalent dose administered is at least 30 Gy. Many of the large trials have used 5x5 Gy of RT immediately followed by surgery. While convenient for the patient and proven to be efficient, this short schedule does not allow to obtain downsizing and downstaging while the large dose per fraction can induce considerable early and late toxicity. The combination of long term RT schedules with chemotherapy initiated in the setting of unresectable rectal cancer and was subsequently introduced in both adjuvant and neoadjuvant therapy of locally advanced resectable disease. In the neoadjuvant setting, numerous phase II trials using CRT have shown a promising response while acute toxicity was generally limited. This prompted the initiation of randomized trials comparing neoadjuvant RT alone versus neoadjuvant CRT. In two recently published trials, preoperative treatment arms encompassing either 45 Gy of RT alone or 45 Gy of RT combined with 5-FU based chemotherapy were included.[2,3] Both trials arrived at very similar conclusions: compared to RT alone, preoperative CRT significantly improved tumor response and local

control while no benefit on overall survival was seen and acute toxicity was moderately increased. Several conclusions can be drawn based on the currently available data. First, patients should receive a tailored multimodal approach. In resectable cancer without possibly compromised CRM as evaluated on preoperative MRI and at a distance from the sphincter apparatus, a short course of preoperative RT is sufficient. When sphincter preservation and/or a possibly compromised CRM is an issue, neoadjuvant CRT with a 6-8 weeks long waiting period before surgery should be initiated. Several questions remain, however, and further improvements in outcome are mandatory. In the surgical domain, important work remains to be done in rectal amputation technique. Several clinical studies have demonstrated that even after expert TME, rectal amputation is associated with a significantly higher rate of invaded CRM and local recurrence. A likely explanation is the 'coning in' at the level of the funnel shaped pelvic floor. Wide cylindrical excision with resection of the pelvic floor musculature might result in a lower recurrence rate, but necessitates flap closure of the wound defect.[4] The effect of tumor downstaging on the sphincter preservation rate is a matter of debate. Many surgeons are reluctant to change a pre-therapy judgement that a patient requires rectal amputation after observation of a good clinical response; future studies will have to determine whether this decision is safe. Improvements in tumor response seem highly desirable, since even with modern CRT regimens the percentage of pCR rarely exceeds 20%. In keeping with the results obtained in the setting of metastatic disease, the addition of targeted agents could enhance the efficiency of neoadjuvant CRT. The anti-VEGF agent

bevacizumab has shown promising synergy with RT in preclinical models and in a phase I study.[5] The radiosensitizing effect of bevacizumab is based on radiosensitizion of tumor-associated endothelial cells, and on normalization of the tumor vasculature and microenvironment resulting in an increase in tumor oxygenation and radiosensitivity. Third, anti-VEGF agents can reduce the number of circulating endothelial cells and progenitor cells leading to the inhibition of recurrent tumor growth after irradiation.[6] Moreover, since RT is known to induce expression of angiogenic mediators, bevacizumab could inhibit the growth of micrometastases. Phase II trials incorporating targeted agents into neoadjuvant CRT regimens for rectal cancer are underway. The observed lack of benefit on overall survival in the trials comparing neoadjuvant RT alone versus CRT, suggests that many patients harbor systemic disease from onset that is insufficiently addressed in the early stages of therapy. Intensification of the RT effect has been sought after by changes in the dose, fractionation, and mode of delivery of the ionizing radiation. Excellent results were obtained in early rectal cancer using a combination of external RT with endocavitary high dose RT.[7] A number of authors have used hyperfractionated (i.e., more than one dose daily) or hyperfractionated accelerated RT (HART) in an effort to increase efficiency and reduce late toxicity.[8,9] The biological rationale of accelerating RT is that the effectiveness of ionizing radiation increases with a higher dose rate by the prevention of malignant cell repopulation and sublethal damage repair between fractions. On the other hand, since the size per fraction is the main determinant of late radiation effects, hyperfractionation is aimed at reducing late toxicity

while achieving a similar tumor control.
In **chapter four**, a clinical study is presented comparing neoadjuvant CRT with neoadjuvant HART in rectal cancer patients. All patients underwent nerve sparing TME. In patients who underwent sphincter sparing surgery, the incidence of anastomotic leakage was low in both groups. Experimental studies have shown that even large doses of preoperative RT do not adversely affect colonic anastomotic healing provided only one limb of the anastomosis is irradiated.[10] The low anastomotic leakage rate in our series can be attributed to the liberal use of a temporary ileostomy, a practice shown to reduce the leakage rate and to mitigate the consequences of leakage following TME.[11,12] In contrast to what would be expected theoretically, HART was associated with a significantly higher early and late complication rate including radioenteritis and rectovaginal fistula. As expected, the pathological response rate was significantly higher in the CRT group. This difference is probably mainly due to the difference in waiting period following completion of neoadjuvant therapy (3.5 days in the HART group and 42.5 days in the CRT group). Interestingly, almost one in five irradiated tumors showed a distinct mucinous differentiation, a finding previously described.[13,14] Selection of mucin producing clones known to be relatively radioresistant could explain this finding.[15] We found a better disease free survival in patients treated with CRT, but the retrospective nature of the study and the difference in mean follow up time limit the relevance of this finding.

Both RT and chemotherapy induce toxicicity, and early deter-

mination of tumor response could prevent unnecessary harm to a patient receiving ineffective therapy. Moreover, with the introduction of angiogenesis agents into the multimodal management of rectal cancer patients, early demonstration of a vascular response would be of interest both in preclinical models, drug development and patient monitoring (RECIST). Radioresponsiveness is determined by a complex set of variables including patient characteristics (age, nutritional status, medication, comorbidity), tumor related factors (size, histology, tumor grade), and treatment-related variables (treated volume, field size, anatomic prescription point, total dose, dose per fraction, concomitant chemotherapy). Additionally, inherent genetic factors determine radioresponsiveness and genomic analysis has been suggested as a tool to predict radiation response.[16] Functional imaging allows visualisation and quantification of therapy effects before changes in tumor volume occur. Metabolic (functional) positron emission imaging using radioactively labelled tracers such as 18F- fluorodeoxyglucose (FDG) has been used to determine therapy response in cancer patients.[17] There are several methods for evaluation of FDG-PET data. The simplest is qualitative comparison of the location, size and intensity of image 'hot spots' by calculation of the standardised uptake value (SUV)for FDG as the product of the ratios: (activity in the lesion / total injected activity) x (subject size / lesion volume). More complex methods include dynamic PET data acquisition with calculation of the rate constant of FDG influx (Ki) using the Patlak plot method. Several drawbacks are associated with FDG-PET imaging as a tool to assess tumor response. First, the repeatability of the method is uncertain, since the methods to

calculate the SUV have not been standardized.[18] According to the RECIST criteria, there needs to be at least a 25% measured increase in unidimensional size to count as progressive disease (PD), and a 30% measured decrease to count as partial response (PR). It is therefore imaginable that changes in SUV are interpreted as PD or PR, while in reality they are the result of poor test repeatability. Second, while PET imaging is highly sensitive, specificity is limited by the fact that inflamed tissue, cardiac and contracting skeletal muscle, nervous tissue, and brown fat will also accumulate FDG. Third, since there is no demonstrated link between glucose metabolism and vascular physiology, the method is not well suited to specifically study vascular effects of anticancer therapy. Magnetic resonance imaging offers a superior spatial and contrast resolution, and has evolved as the standard of care in preoperative imaging of rectal cancer allowing accurate prediction of surgical margin involvement.[19] In addition to MRI examination using static images, dynamic studies following injection of a bolus of paramagnetic CA allows morphological as well as functional microvascular characterization of tumor tissue.[20] In **chapter five**, we examined the use of DCE-MRI to assess early response to RT in a rat colorectal model. Since we were mainly interested in microvascular permeability changes, a blood pool CA was chosen. Although the MW of P792 is only 6.47 kDa, the 3D structure of the molecule with its four extending branches causes it to behave as a macromolecule. The results demonstrate that P792 does not enhance normal tissue while a slowly increasing enhancement was seen in neoplastic tissue, with a maximal rate and extent of signal enhancement in the vascular tumor rim. This indicates that

P792 is a suitable DCE-MRI contrast agent in assessment of tumor microvascular properties. The signal intensity data were subsequently translated into CA concentrations and fitted in a two compartment pharmacokinetic model according to Tofts. We have used a dose and fractionation identical to those clinically used, and found the endothelial transfer constant (Ktrans) to decrease significantly following RT. When using a BPCA, Ktrans is a measure of vascular permeability and is relatively independent on the tumor blood flow. However, it cannot be excluded that the observed decrease in Ktrans following RT results from changes in the density of microvessels and from changes in blood flow as well as from a decreased permeability. Since animals were followed longitudinally, we were unable to determine MVD (requiring excision of the tumor) before and after RT in the same animal. However, since no significant difference in MVD was observed between irradiated and control animals, we assume that the observed decrease in Ktrans is mainly due to a decrease in vessel permeability. The fact that we were unable to demonstrate a correlation with MVD illustrates the functional rather than morphological nature of DCE-MRI characterization of the tumor vascular bed. Although the pharmacokinetic data obtained with the used DCE-MRI method seem robust, several limitations are inherent to the method. First, data are modelled according to a mathematical model and as such are based on a number of assumptions. It is assumed that the CA is instantaneously homogeneously mixed in the vascular compartment, that no shunting is present, and that a single arterial input can be identified. These assumptions are of course overt simplifications of the physiological reality. Second, the assumed

linear relation between signal intensity and CA concentration does not always hold and this can have a major impact on the result of modelling.[21] Third, most of the MMCA and BPCA used in preclinical DCE-MRI vascular imaging are not (yet) available for clinical use. Nevertheless, an increasing number of papers has used parameter modelling of DCE-MRI data in clinical studies of anticancer therapy response assessment.[22-24] The tumor microvascular bed has been identified as an important target and mediator of RT effects in recent years, and some authors have even suggested the endothelial cell response to be the primary mediator of RT effect.[25,26] The effect of RT on endothelial cell permeability is incompletely understood. Published experimental data suggest that, depending on the dose and fractionation of RT and the timing of permeability measurement, neovascular permeability can be either increased or decreased. Large, single RT doses disrupt the endothelial lining and cause a short-term increase in endothelial permeability while smaller or fractionated doses tend to decrease permeability.[27] The molecular mechanisms whereby fractionated RT decreases endothelial permeability are unknown, but could include changes in the expression of cell-cell adhesion molecules, claudins, occludins, and other components of intercellular tight junctions.[28,29] Also, radiation was shown to induce expression of alpha(v)beta(3) integrin in cultured endothelial cells.[30] In vitro, irradiated endothelial cells increased proliferation and migration of vascular smooth muscle cells by pathways including increased expression of transforming growth factor (TGF).[31] Interesting clinical data were obtained by Baeten et al., who examined angiogenesis related parameters in pre- and post therapy

biopsy samples from rectal cancer patients undergoing neoadjuvant RT or CRT.[32] They found an inhibition of endothelial cell proliferation after RT, associated with an increased expression of VEGF and adhesion molecules together with increased leucocyte infiltration. We also found an increase in VEGF expression in irradiated animals compared to controls, and this provides a theoretical rationale to combine RT with angiogenesis targeting therapy. We combined DCE-MRI with invasive pO2 measurements and found a significant increase in pO2 in the peripheral region of the tumor after RT. The available evidence concerning the effect of fractionated radiation on tumor oxygenation suggests that either an increase or a decrease in oxygenation can be observed depending on RT dose, fractionation, tumor histology, and timing of pO2 measurement. Enhanced oxygenation shortly after fractionated radiotherapy has been previously reported and is the net result of changes in both oxygen delivery (reduced interstitial pressure, increased flow) and oxygen consumption (decreased cell density).[33,34]

There has been a long standing interest in the relationship between tumor oxygenation and the response of solid tumors to RT.[35] Clinical studies using needle electrode measurements of tumor oxygenation before therapy have consistently shown tumor hypoxia to be associated with resistance to therapy (RT, chemotherapy, and surgery), increased metastatic phenotype, and worse prognosis.[36] The molecular background of hypoxia induced radioresistance consists in the fact that when present intracellularly, molecular oxygen binds to DNA fragments and renders RT induced damage irreversible. Efforts to increase tumor oxygenation by hyperbaric oxygen, radiosensitizers, and

bioreductive agents have met with a limited clinical success rate.[37] Since many cancer patients suffer from anemia, administration of EPO might improve tumor oxygenation and hence therapy outcome. Preclinical models have shown that administration of EPO enhances tumor oxygenation and improves response to RT.[38,39] Conflicting results have been obtained, however, in clinical trials studying the effect of EPO administration on RT efficiency and survival.[40] While randomized trials in esophageal [41] and endometrial [42] cancer have shown a survival benefit in EPO treated patients, studies in head and neck [43] and breast cancer [44] patients demonstrated an adverse effect of EPO administration on local disease control and long term outcome. These results combined with the demonstration of the EPO receptor on endothelial cells and several tumor cell types, have lead to research into the direct effects of EPO on angiogenesis and tumor growth (Fig 7.1).

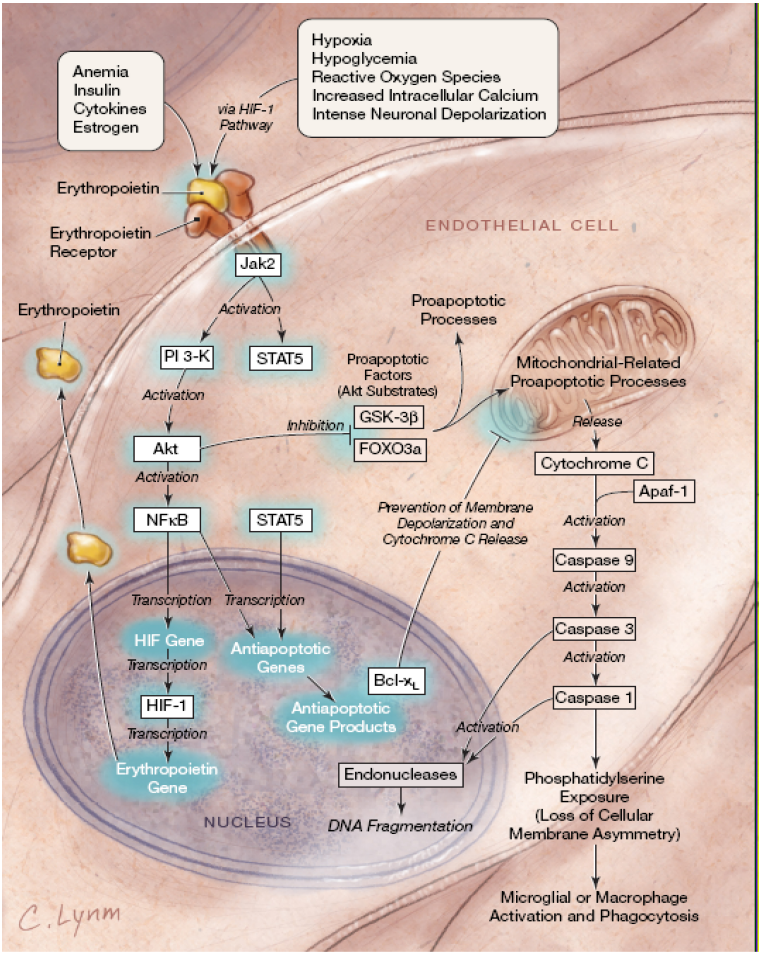

Figure 7.1: Erythropoietin (EPO) and the EPO receptor (EPOR) prevent apoptosis and cellular inflammation and preserve cellular integrity through a series of signalling cascades including Janus-tyrosine kinase 2 (Jak2),phosphoinositide 3 kinase (PI 3-K), and protein kinase B (Akt). Reproduced with permission from Maiese K, Li F, Chong Z. New Avenues of Exploration for Erythropoietin. JAMA 2005;293:90-95.

At present, the results from preclinical models are conflicting.[45] Moreover, in vitro data from Belenkov et al. suggested that EPO induces resistance to ionizing radiation and chemotherapy, an effect that was reversed by administration of an inhibitor of tyrosine kinase Jak2, the main intracellular target of EPO receptor binding.[46] There are at present no in vivo data regarding the effect of EPO on radioresponsiveness of the microvascular bed. In **chapter six**, we examined the modulation by recombinant human EPO of the response of colorectal microvessels to fractionated RT. Immunohistochemically, intense expression of the EPO receptor on neoplastic cells and tumor associated endothelium was observed. In this non anemic rat colorectal cancer model, morphological and functional microvascular differences after RT were present between EPO treated and control animals. Specifically, EPO administration partially inhibited the RT induced reduction in microvascular permeability noted in the experiments described in chapter five. Moreover, microvessels in the EPO group were larger in diameter while microvascular complexity was less in the central region (where EPO receptor expression was higher) of EPO treated tumors. Microvessel density, however, was not different between EPO treated and control animals. We also found a reduced expression of VEGF in EPO treated animals compared to controls while no differences were present in the expression of HIF-1 alpha, Bcl-2, or Bax. Taken together, these results suggest a remodelling of colorectal cancer microvessels by EPO, as already suggested by Tovari et al.[47] The effect of EPO on microvessels has also been noted clinically. In patients with ischemic heart disease, administration of EPO resulted in an increase in circu-

lating bone marrow derived endothelial progenitor cells resulting in enhanced neovascularisation.[48] The molecular mechanisms of the interaction between exogenous EPO, tumor associated endothelial cells, and tumor stroma remain to be elucidated.

7.1 Conclusion and Future Perspectives

Neoadjuvant multimodal therapy is the standard of care in locally advanced resectable rectal cancer. When sphincter preservation or possible CRM compromise is important, neoadjuvant chemoradiation with a 6-8 weeks waiting period before surgery results in increased tumor response and enhanced sphincter preservation although overall survival is not different. Compared to chemoradiation, neoadjuvant HART is less effective in terms of tumor response, toxicity, and disease free survival.
DCE-MRI with the blood pool contrast agent P792 can successfully image and quantify physiological properties of the neoplastic vascular bed. Administration of 5x5 Gy of RT results in a significant decrease of capillary permeability and results in reoxygenation of tumor tissue. Exogenous EPO enhances tumor oxygenation, prevents the RT induced decrease in capillary permeability and results in remodelling of the microvascular bed.
Future work of our group will include molecular MRI to image angiogenesis using a paramagnetic CA designed to target alfa(v)beta(3) integrin, expressed almost exclusively by activated endothelium. In order to correlate functional imaging with a gold standard, in vivo fluorescence microscopy will be used to study capillary permeability in a dorsal skinfold cham-

ber using CA that are tagged with a fluorescent marker. Using this in vivo imaging technology and CC531 tumor cells expressing green fluorescent protein (GFP), early stages of angiogenesis and hepatic metastasis will be studies as well as the effects of various therapeutic strategies targeting angiogenesis.

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Chapter 8

Summary

The aim of this work is to contribute clinical and experimental data on the neoadjuvant multimodality therapy of resectable rectal cancer. In **chapter three**, a systematic review of the literature is provided. While it is clear that technical improvements (TME) have dramatically lowered local recurrence rates following rectal cancer surgery, patients with cT3 disease benefit from some form of neoadjuvant therapy, as evidenced by the Scandinavian and Dutch rectal cancer trials. When the CRM is not at risk and the tumor is at a distance from the sphincter apparatus, neoadjuvant RT alone immediately followed by surgery is appropriate. However, when the CRM is at risk or possible sphincter invasion is evident, neoadjuvant CRT followed by a waiting period of at least 6-8 weeks is indicated in order to achieve downsizing and downstaging. Two large randomized trials encompassed treatment arms comparing neoadjuvant RT

alone versus neoadjuvant CRT. The addition of chemotherapy results in a superior tumor response, translating into a lower local recurrence rate and better local control at the expense of moderately increased toxicity. Overall survival is not improved by the addition of chemotherapy. Neoadjuvant CRT results in a pCR in up to 20% of patients. With the currently available imaging techniques, achieving a clinical CR does not preclude surgery since residual cancer can not be excluded with certainty. Perspectives for further improvement include the addition of angiogenesis targeting agents to neoadjuvant therapy regimens and early intensification of chemotherapy in order to improve overall survival.

Several modifications of the RT regimen have been described in order to further increase tumor response while avoiding early and late toxicity to normal tissue. In **chapter four**, we compared neoadjuvant CRT with neoadjuvant HART in a clinical study of resectable distal rectal cancer. Therapy consisted of either 45 Gy in 25 fractions of 1.8 Gy with 5-FU based chemotherapy followed by TME after 6-8 weeks or 41.6 Gy in 26 fractions (BID) of 1.6 Gy followed by immediate TME. We found that CRT was associated with more sphincter preserving procedures (74% (HART) versus 83.5% (CRT), p=0.013). Tumor response was better in the CRT group, with a pCR observed in 4% (HART) versus 18% (CRT) of the resected specimens (p=0.002). Although no differences were observed in the anastomotic leak rate, both early and late toxicity were significantly more pronounced after HART. Disease free 5 year survival was 51% (HART) versus 62% (CRT), p=0.037 while no differences were present in local recurrence rate or overall survival.

Early assessment of tumor response is important in drug development, clinical trials, and follow up of individual patients. Functional imaging of the neoplastic vascular bed allows early visualization and enhancement of neoadjuvant therapy. In **chapter five**, we studied DCE-MRI with P792, a BPCA to monitor RT induced microvascular changes in a rat CC531 colorectal tumor model. A two compartment pharmacokinetic model was used to generate parametric maps of the endothelial transfer constant (Ktrans) and the fractional EES (Ve). Imaging before and 5 days after RT was combined with oxygenation and flow measurements. Microvessel density and VEGF expression were compared with a control group of non irradiated tumors. We found a significant reduction in Ktrans and EES fraction following 5x5 Gy of RT, indicating reduced capillary permeability. Although tumor oxygenation was increased after RT, VEGF expression was significantly higher in RT treated rats compared to controls while pimonidazole hypoxia score and MVD were not significantly different. After RT, no significant correlation was found between DCE-MRI parameters and histological parameters. An inverse correlation was seen after RT between pO2 and Ktrans ($r = -0.57$, $p = 0.08$) and between pO2 and Ve ($r = -0.65$, $p = 0.04$). These data indicate that DCE-MRI could be valuable to visualize and quantify early response to anti-angiogenic therapy, including RT.
Since solid tumors inevitably are hypoxic and the degree of hypoxia is related to RT response and outcome, several approaches to increase tumor oxygenation have been described. In **chapter six**, we studied the effect of exogenous recombinant EPO on the response of a colorectal tumor to RT using a similar rat

model. Tumors (one control group and one EPO treated group) were irradiated with 5x5 Gy or RT. Before and 5 days after RT, DCE-MRI was performed with compartmental modelling allowing to generate parametric maps of blood volume, permeability surface product (PS), and plasma flow. Imaging was combined with pO2 and laser doppler flow (LDF) measurements. After the second set of measurements, tumors were analysed for MVD, vessel diameter, and fractal dimension (MFD), a measure of vascular geometric complexity. Expression of VEGF, HIF-1 alpha, Bax, and Bcl-2 was determined immunohistochemically. We found that RT significantly reduced PS in control rats, but not in rhEPO treated rats. Oxygenation was significantly better in rhEPO treated animals, and RT induced a spatially heterogeneous reoxygenation in both groups. Microvessel diameter was significantly larger in rhEPO animals, while MFD was lower in the tumor core. VEGF expression was significantly lower in the rhEPO group. No differences were observed in HIF-1 alpha, Bax, or Bcl-2. These results suggest that exogenous EPO administration results in spatially heterogeneous modulation of RT effects on tumor microvessels. Direct effects of rhEPO on neoplastic endothelium are likely to explain these findings in addition to indirect effects induced by increased oxygenation. The exact molecular mechanisms whereby EPO affects endothelium during RT remain to be elucidated.
Future work from our group will include molecular angiogenesis imaging using a alpha(v)beta(3) integrin specific MRI contrast agent. Furthermore, in vivo fluorescent microscopy will be used 1. to validate MRI contrast agents as a tool to measure capillary permeability and 2. to study early tumor angiogenesis and

metastatic events using fluorescently labelled cancer cells.

Chapter 9

Samenvatting

Het doel van dit werk is zowel een klinische als experimentele bijdrage te leveren in de multimodale behandeling van het rectumcarcinoom. In **hoofdstuk drie** wordt een systematisch overzicht van de literatuur gepresenteerd. Het is duidelijk dat een nauwkeurige chirurgische techniek (total mesorectal excision of TME) het voorkomen van lokale recidieven na rectumresectie sterk heeft verminderd. Toch is voor een cT3 tumor een vorm van neoadjuvante behandeling aangewezen, zoals aangetoond door de Scandinavische and Nederlandse studies terzake. Indien de circumferentiële resectiemarge (CRM) niet bedreigd is en de tumor zich op afstand van de sluitspier bevindt, volstaat neoadjuvante radiotherapie (RT) met onmiddellijke chirurgie. Indien echter de CRM bedreigd is en/of de tumor zich dicht bij de sluitspier bevindt, is een lange kuur RT gecombineerd met chemotherapie aangewezen gevolgd door chirurgie na 6-8 weken

teneinde de tumor toe te laten in grootte (en stadium) af te nemen. Twee grote klinische studies vergeleken neoadjuvante RT alleen met neoadjuvante chemoradiatie (CRT). Het toevoegen van chemotherapie bleek de tumorrespons op RT te verhogen, hetgeen zich vertaalde in een groter aantal sphinctersparende ingrepen en een lagere kans op lokaal recidief. De acute toxiciteit was evenwel wat hoger, terwijl er ook geen effect op de globale overleving kon worden aangetoond. Neoadjuvante CRT resulteert in een pathologische volledige respons (pCR) in ongeveer 20% van de patiënten. Indien na CRT een klinische volledige respons wordt vastgesteld, blijft chirurgie noodzakelijk vermits de huidig beschikbare technologie niet toelaat residuele tumor met zekerheid uit te sluiten. De repons op CRT zal mogelijk nog verbeteren door toevoegen van angiogenese remmers aan de chemotherapie; daarnaast zal er mogelijk een rol zijn voor vroege intensificatie van de chemotherapie om de overleving op lange termijn te verbeteren.
Er zijn verschillende methoden beschreven om de tumorrespons op RT te verbeteren terwijl de neveneffecten op de normale weefsels beperkt blijven. In **hoofdstuk vier** vergeleken we in een klinische studie neoadjuvante CRT met neoadjuvante gehyperfractioneerde geaccelereerde radiotherapie (HART) in de behandeling van het distaal rectumcarcinoom. De behandeling bestond uit 45 Gray (Gy) in 25 fracties van 1.8 Gy met 5-FU gebaseerde chemotherapie gevolgd door TME na 6-8 weken of 41.6 Gy in 26 fracties (twee daags) van 1.6 Gy gevolgd door onmiddellijke TME. We vonden dat CRT leidde tot meer sphinctersparende ingrepen (74% (HART) versus 83.5% (CRT), p=0.013). De tumorrespons was beter in de CRT group, met

een pCR in 4% (HART) versus 18% (CRT) van de verwijderde specimens (p=0.002). Hoewel geen verschillen werden genoteerd in anastomotisch lek, waren zowel vroegtijdige als late nevenwerkingen veel meer uitgesproken na HART. De ziektevrije 5 jaars overleving was 51% (HART) versus 62% (CRT), p=0.037 terwijl geen verschillen werden vastgesteld in het aantal lokale recidieven of in de globale 5 jaars overleving.
Vroegtijdige bepaling van het antwoord op neoadjuvante therapie is van groot belang voor zowel ontwikkeling van nieuwe antitumorale behandelingen, klinische studies, en beleid bij de individuele patiënt. Functionele beeldvorming laat toe, vroegtijdig het antwoord van het tumorvaatbed op therapie te visualiseren en te quantifiren. In **hoofdstuk vijf** wordt dynamische contrast MRI (DCE-MRI) met P792, een blood pool contraststof (CA) aangewend om de microvasculaire respons op RT te bestuderen in een rat CC531 colorectaal tumormodel. De DCE-MRI data werden verwerkt in een twee compartiment farmacokinetisch model met genereren van parametrische kaarten van de endotheliale transferconstante (Ktrans) en de fractie van de extravasculaire extracellulaire ruimte (EES). De beeldvorming werd gecombineerd met meting van weefseloxygenatie en uitgevoerd voor en vijf dagen na 5x5 Gy RT. Daarnaast werden microvaat dichtheid (MVD) en expressie van de vasculaire endotheliale groeifactor (VEGF) vergeleken met een groep niet bestraalde tumoren. We vonden een significante reductie van Ktrans en van de EES fractie, wijzend op een gedaalde capillaire permeabiliteit na RT. Hoewel de weefseloxygenatie toenam na RT, bleek de expressie van VEGF hoger in bestraalde tumoren terwijl geen significante verschillen werden genoteerd

in MVD of expressie van de hypoxiemerker pimonidazole. In de bestraalde tumoren kon geen correlatie worden aangetoond tussen de DCE-MRI parameters en de MVD. Er werd een inverse correlatie gezien tussen pO2 and Ktrans (r = -0.57, p = 0.08) en tussen pO2 and Ve (r = -0.65, p = 0.04). Deze resultaten tonen aan dat DCE-MRI met een macromoleculaire CA nuttig kan zijn in de vroegtijdige visualisatie en quantificatie van anti-angiogenese therapie waaronder RT.
Solide tumoren vertonen onvermijdelijk een zeker mate van hypoxie, en het verband tussen hypoxie en zowel respons op RT als overleving zijn aangetoond in meerdere klinische studies. Er bestaat dan ook belangstelling in behandelingen die de tumoroxygenatie doen toenemen met als doel de respons op RT te verbeteren. In **hoofdstuk zes** werd het effect van exogeen toegediend erythropoietine (EPO) onderzocht op de respons van tumor microvaten op RT. Een CC531 tumor werd geïnduceerd bij de rat waarna een controlegroep en een EPO behandelde groep 5x5 Gy RT ondergingen met DCE-MRI zowel voor als 5 dagen na de RT. De DCE-MRI data werden gefit in een meer complex farmacokinetisch model dat toelaat om naast de capillaire permeabiliteit en het bloedvolume ook de plasma flow te berekenen. De beeldvorming werd gecombineerd met oxygenatiemeting en immuunhistochemische bepaling van MVD, diameter, fractale dimensie (MFD) van de microvaten (een maat voor de geometrische complexiteit), en expressie van VEGF, hypoxie induceerbare factor (HIF) 1 alpha, Bcl-2, en Bax. We vonden een significante reductie van capillaire permeabiliteit na RT in de controle groep, maar niet in de EPO behandelde groep. De weefseloxygenatie was significant beter in de EPO groep, en

RT leidde tot een ruimtelijk heterogene toename van de pO2 in beide groepen. Toediening van EPO leidde tot morfologische verschillen met de controle groep na RT (grotere diameter, lagere MFD) terwijl geen verschillen in MVD werden aangetroffen. De expressie van VEGF was significant lager na RT in de EPO behandelde groep, terwijl geen verschillen werden gevonden in expressie van HIF-1 alpha, Bcl-2, en Bax. Deze resultaten suggereren een ruimtelijke modulatie van tumorale microvaten door EPO, die waarschijnlijk zowel door rechtstreekse effecten op het endotheel als door indirecte effecten (door gewijzigde oxygenatie) te verklaren zijn. De moleculaire mechanismen waardoor EPO het antwoord van tumorale microvaten op RT wijzigt dienen nader te worden opgehelderd.
Toekomstig werk binnen onze groep omvat onder meer moleculaire MR beeldvorming van de angiogenese met een CA gericht tegen het alpha(v)beta(3) integrine. Daarnaast zal in vivo fluorescentiemicroscopie worden gestart teneinde 1. over een gouden standaard te beschikken in verband met evaluatie van capillaire permeabiliteitsmeting met MRI; en 2. vroege stappen van (tumorale) angiogenese te bestuderen en het effect van antiangiogene behandelingen. Daarbij zal worden gebruik gemaakt van tumorcellen die green fluorescent protein (GFP) tot expressie brengen.

BED	Biologically Equivalent Dose
BPCA	Blood Pool Contrast Agent
CA	Contrast Agent
cCR	Clinical Complete Response
CMT	Combined Modality Therapy
CRM	Circumferential Resection Margin
CRT	Chemoradiotherapy
CT	Chemotherapy
Da	Dalton
DCE-MRI	Dynamic Contrast Enhanced Magnetic Resonance Imaging
EES	Extravascular Extracellular Space
EPO	Erythropoietin
Gd	Gadolinium
Gy	Gray
HART	Hyperfractionated Accelerated Radiotherapy
kDa	Kilodalton
Ktrans	Endothelial Transfer Constant
LR	Local Recurrence
MFD	Microvascular Fractal Dimension
MMCA	Macromolecular Contrast Agent
MRI	Magnetic Resonance Imaging
MVD	Microvessel Density
MW	Molecular Weight
pCR	Pathological Complete Response
PD	Progressive Disease
PET	Positron Emission Tomography
PR	Partial Response
PS	Permeability Surface Product
RECIST	Response Criteria in Solid Tumors
rhEPO	Recombinant Human Erythropoietin
RT	Radiotherapy
SUV	Standardised Uptake Value
TME	Total Mesorectal Excision
Ve	Fractional EES Volume
VEGF	Vascular Endothelial Growth Factor
Vp	Fractional Vascular Volume

Curriculum Vitae

1. Personal data

   - Name: Ceelen
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   - Nationality: Belgian
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3. Training

   - 1980-1985 : Humaniora Latin-Greek, St Barbaracollege Ghent
   - 1985-1992 : Medical School Study, Ghent University medical school
   - Foreign training: Hospital Del Mar, Barcelona (3 months)

- 1992-1998 : Training in General Surgery
  UZ Ghent (Prof B. de Hemptinne)
  Canisius Wilhelmina Ziekenhuis, Nijmegen, Netherlands (D. Deleu)
  Heilig Hartziekenhuis, Oostende, Belgium (D. Goegebuer)

4. Diplomas and Postgraduate Courses

   - 7/1992 Doctor in Medicine, with high honours (magna cum laude)
   - 6/1992 ECG-certificate (Prof. Clment)
   - 1994 US medical license (ECFMG part I en II), Paris, passed
   - 1995 Postgraduate course intensive care medicine
   - 1996 Postgraduate course ESOT, Milano
   - 1998 Postgraduate course biomedical statistics
   - Oct 1998 Licenced as surgeon (RIZIV/INAMI)
   - 1999 Postgraduate course Surgical Infections (SIS-Europe, Halle, Germany)

5. Languages

   Mother language: Dutch (Nederlands). Excellent written and oral knowledge of English and French; good written mastery of German and Spanish.

6. Current position

   - Joint Head of Clinic, Department of Abdominal Surgery (Prof B. de Hemptinne),
     Division of Gastrointestinal Surgery (Prof P Pattyn),
     UZ Ghent

- Docent Hogeschool Gent

7. Professional Interests

   - Surgical oncology, colorectal surgery, peritoneal carcinomatosis
   - Scientific writing and publishing, evidence based medicine
     Editor of Acta Chirurgica Belgica; Reviewer for Ann Surg Oncol, Int J Radiat Oncol Biol Phys, Eur J Cancer, J Appl Physiol
   - Experimental work: rat colorectal cancer model with study of RT/hyperthermia; use of DCE-MRI with macromolecular contrast agents to monitor microcirculation
   - Clinical studies: HOLA trial (open versus laparoscopic incisional hernia repair), industry sponsored studies (Pegasus, Alvimopan, Promise)
   - Information technology and statistics (experience with SPSS, GraphPad prism, SigmaStat, Linux, Apache webserver and hosting, Windows 2000/XP OS and applications)

8. Professional Memberships

   - ESSR (European Society for Surgical Research): Secretary General, webmaster
     Congress President ESSR May 28-31 2003, Ghent, Belgium
   - ESSO (European Society for Surgical Oncology): board member

- RBSS (Royal Belgian Society of Surgery): board member
- BGES (Belgian Group for Endoscopic Surgery): board member
- BSSO (Belgian Society for Surgical Oncology): board member
- BGDO (Belgian group for Digestive Oncology)
- VVGE (Vlaamse vereniging voor Gastroenterologie)
- EORTC GI group
- Cochrane Colorectal Cancer Group

9. Prizes

    - Laureate Grant Vlaamse Vereniging voor Gastroenterologie 1999
    - Laureate Junior prijs Royal Belgian Society for Surgery 2000
    - Laureate cancer research grant Foundation Rik en Nel Wouters 2001
    - Laureate Duprez prize 2001, Royal Belgian Society for Surgery

10. Other Interests

    - piano, poetry, Mozart's operas
    - mountain bike, running

11. Mottos

    - *"Never for me the lowered banner, never the lost endeavour"* (Shackleton)
    - *"aien aristeuein kai upeirochon emmenai alloon"* (Homeros, Iliad)

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